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(54) Title: VECTOR

(57) Abstract: A autoregulatory expression vector is provided which comprises a nucleic acid sequence encoding reverse tetracycline transactivator which binds to a tet operon containing promoter in the presence of doxycycline thereby inducing expression of a protein of interest. The vector finds application in gene therapy in the treatment of chronic conditions displaying relapsing symptoms.

VECTOR

The present invention relates to a vector and uses of the vector in medicine, with particular application in gene therapy.

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The development of transcriptionally controlled systems which function in eukaryotic cells are important for achieving regulated gene expression in gene therapy. The tetracycline system for regulated gene expression was originally developed as a two vector 'off' system in which a chimeric tetracycline transactivator (tTA), composed of tetR and VP16 is constitutively expressed from one vector, and binds to a tetracycline operon (tetO) containing promoter in a second vector inducing gene expression (Gossen & Bujard, *Proc. Natl. Acad. Sci. USA* **89** 5547-5551 (1992)).

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When tetracycline is added it binds tTA and induces a conformational change which prevents it from binding to the tetO and thus switches off gene expression. The 'off' system displays regulated expression in excess of 1000-fold when the two vectors are stably transfected into HeLa cells. Several studies have since reported adaptations of the original tetracycline 'off' system which facilitate application in gene therapy settings.

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One variation involves combining the components of the two vectors in a single self-contained vector, which avoids the need to deliver two vectors to a single cell, this combination has been reported with both retroviral (Paulus *et al*, *J. Virol.* **70** 62-67 (1996); Lindermann *et al*, *Mol. Med.* **3** 466-476 (1997)) and plasmid vectors (A-Mohammadi *et al*, *Gene Therapy* **5** 76-84 (1998)). Another modification has been expressing tTA under the control of a tetO promoter. In this autoregulatory format the tTA regulates its own expression, in the absence of tetracycline the tTA is able to interact with the tetO promoter and can therefore upregulate its own expression in a positive feedback loop. When tetracycline is added to the system the tTA can no longer interact with the promoter and so tTA expression is down regulated. The

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advantage of autoregulated expression of tTA is low level expression of tTA in the presence of tetracycline, which should reduce toxic effects of constitutively expressed tTA, and higher levels of inducible gene expression when tetracycline is removed.

5 The autoregulated expression of tTA was first adopted in a two vector system (Shockett *et al.*, *Proc Natl Acad Sci USA* **92**: 6522-6526 (1995)) and was later incorporated into self-contained vectors (A-Mohammadi & Hawkins *Gene Therapy* **5**: 76-84 (1998); Hofmann *et al.*, *Proc Natl Acad Sci*; **93**: 5185-5190 (1996)). In the 'off' system tTA autoregulation results in high level expression of tTA in the absence
10 of antibiotic. However, it has been postulated that tTA may have a 'squelching' effect at high intracellular levels (Gossen & Bujard, *Proc Natl Acad Sci USA*; **89**: 5547-5551 (1992)). Indeed this view was supported by a study which indicates that continued high level tTA expression from an autoregulatory vector is detrimental to cell functions including growth and the cell cycle (Gallia & Khalili, *Oncogene* ; **16**: 15 1879-1884 (1998)). By contrast cells maintained in the presence of tetracycline displayed normal growth characteristics.

20 The toxic effects of tTA are usually attributed to potential 'squelching' effect of the VP16 component, but the tetR component could also contribute to these effects. A study examining tetR expression in tomato plants, demonstrated that high levels of the *Tn10* encoded tetR above a threshold level which displayed toxic effects including reduced leaf chlorophyll content, leaf size and root dry weight (Corlett *et al.*, *Plant, Cell and Environment* ; **19**: 447-454 (1996)). Interestingly, supplementing the sand with tetracycline significantly reduced the deleterious effects in plants.

25 Autoregulated expression of tTA in self-contained vectors requires tetracycline (Tc) or tetracycline derivatives to switch off the tTA-dependant expression unit. Even though Tc and many Tc derivatives are nontoxic to eukaryotic cells at the low concentrations required to abolish gene expression, their continuous presence is undesirable in a 30 variety of experimental set-ups, for example, in the breeding of transgenic animals and

in gene therapy. Moreover, the induction of gene expression may be slower when an effector needs to be removed, a feature which is disadvantageous in situations where the kinetics of switching on/off gene expression play a role, for example, in developmental processes.

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Gossen and co-workers later developed a two-vector tetracycline 'on' system. Mutation of the tTA produced the reverse tetracycline transactivator (rtTA) which induces gene expression in the presence of doxycycline (Gossen *et al*, *Science* **268** 1766-1769 (1995)).

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The present inventors have developed a system which has been found to demonstrate surprisingly rapid kinetics for switching on/off gene expression. It has been found that rapid on/off switching of gene expression can be achieved using an autoregulated expression vector which utilises the reverse-tetracycline expression unit.

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According to a first aspect of the invention there is provided an autoregulatory expression vector comprising a first nucleic acid construct and a second nucleic acid construct, the first and second nucleic acid construct comprising a promoter sequence and a tet-operator sequence (tetP); wherein, the first nucleic acid construct comprises a first nucleic acid sequence encoding a protein of interest and the second nucleic acid construct comprises a second nucleic acid sequence encoding reverse tetracycline transactivator (rtTA); and wherein each of the first and second nucleic acid sequences are provided with a termination sequence.

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In the present application, the term "autoregulatory expression vector" means any nucleic acid vector encoding a nucleic acid sequence e.g a gene, which when expressed leads to increased or decreased levels of the expression product which up or down regulates further expression of the nucleic acid sequence either through positive or negative feedback.

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As used herein, the term "expression vector" is intended, where appropriate, to mean "autoregulatory expression vector" in accordance with the first aspect of the invention.

5 The term "vector" generally refers to any nucleic acid vector which may be RNA, DNA or cDNA.

10 The term "expression vector" may include, among others, chromosomal, episomal, and virus-derived vectors, for example, vectors derived from bacterial plasmids, from bacteriophage, from transposons, from yeast episomes, from insertion elements, from yeast chromosomal elements, from viruses such as baculoviruses, papova viruses, such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids. 15 Generally, any vector suitable to maintain, propagate or express nucleic acid to express a polypeptide in a host may be used for expression in this regard.

20 Preferably the expression vector is a bacterial plasmid for example the bacterial plasmid pGTRTL or pGTRTC. Constructs of pGTRTL and pGTRTC are shown in Figure 1. Alternatively, the expression vector may be a retroviral vector example M1-LUC-CMV or M2-LUC-CMV. Constructs of M1-LUC-CMV and M2-LUC-CMV are shown in Figure 1.

25 In certain embodiments of the invention, the vectors may provide for specific expression. Such specific expression may be inducible expression or expression only in certain types of cells or both inducible and cell-specific. Preferred among inducible vectors are vectors that can be induced for expression by environmental factors that are easy to manipulate, such as temperature and nutrient additives. Particularly preferred among inducible vectors are vectors that can be induced for expression by changes in the levels of chemicals, for example, chemical additives such as antibiotics. 30 A variety of vectors suitable for use in the invention, including constitutive and

inducible expression vectors for use in prokaryotic and eukaryotic hosts, are well known and employed routinely by those skilled in the art.

5 Recombinant expression vectors will include, for example, origins of replication, a promoter preferably derived from a highly expressed gene to direct transcription of a structural sequence, and a selectable marker to permit isolation of vector containing cells after exposure to the vector.

10 Mammalian expression vectors may comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation regions, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking non-transcribed sequences that are necessary for expression. Preferred mammalian expression vectors according to the present invention may be devoid of enhancer elements.

15 The promoter sequence may be any suitable known promoter, for example the human cytomegalovirus (CMV) promoter, the CMV immediate early promoter, the HSV thymidine kinase promoter, the early and late SV40 promoters or the promoters of retroviral LTR's, such as those of the Rous sarcoma virus ("RSV"), and metallothionein promoters, such as the mouse metallothionein-I promoter. The promoter may comprise the minimum sequence required for promoter activity (such as a TATA box without enhancer elements), for example, the minimal sequence of the CMV promoter (mCMV). Preferably the promoter is a mammalian promoter that can function at a low basal level devoid of an enhancer element.

20 25 Preferably, the promoter is contiguous to the first and/or second nucleic acid sequence. In practice, the promoter lies between the tet operator sequence and the first or second nucleic acid sequence.

5 The tet operator sequence of the expression vector of the first aspect of the invention may comprise seven tet operators located upstream from the sequence of the promoter. It is contemplated that variants, for example, homologues or orthologues, of the promoters described herein are part of the present invention. Preferably, the tet operator sequence is suitably operatively linked to the tet operator sequence.

10 In some situations it may be desirable for the tet operator sequence to be devoid of binding sites for transcription factors which effect the level of basal activity in the expression system. In such situations, the tet operator may be devoid of nucleic acid sequences which bind to the GATA sequence of transcription factors. Moreover, the tet operator which is devoid of GATA binding sites may retain a suitable recognition sequence for the tet repressor (tetR). In this context the transcription factors may be endogenous transcription factors.

15 20 Preferably, the backbone of the expression vector of the first aspect of the invention is derived from a vector devoid of its own promoter and enhancer elements, for example the plasmid vector PGL2. Enhancers are able to bind to promoter regions situated several thousands of bases away through DNA folding (Rippe *et al* *TIBS* 1995; **20**: 500-506 (1995)). In the event of an interaction between enhancer elements of promoters in self contained vectors, rtTA may be prevented from binding to the tetO elements of the tetP, or the expression of rtTA from the adjacent promoter, for example CMV promoter, may in some way be reduced.

25 The expression vectors may also include selectable markers, such as antibiotic resistance, which enable the vectors to be propagated.

In the present application, the promoter sequence and tet-operator sequence are referred to as "tetP".

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The tetP sequence may be adjacent to the nucleic acid sequence of the first and/or second nucleic acid construct. Preferably, the teP sequence is suitably operably linked to the nucleic acid sequence of the first and/or second nucleic acid construct. In practice, the tetP sequence controls the expression of the nucleic acid sequence of the first and second nucleic acid construct. In this situation, in the presence of an effector, rtTA binds to the tet operator sequence inducing expression of the first and second nucleic acid sequence.

The first nucleic acid sequence of the expression vector of the first aspect of the invention may be a reporter transcription unit lacking a promoter region, such as a chloramphenicol acetyl transferase ("CAT") transcription unit. As is well known, introduction into an expression vector of a promoter-containing fragment at a restriction site upstream of the CAT gene engenders the production of CAT activity, which can be detected by standard CAT assays. The application of reporter genes relates to the phenotype of these genes which can be assayed in a transformed organism and which is used, for example, to analyse the induction and/or repression of gene expression. Reporter genes for use in studies of gene regulation include other well known reporter genes including the *lux* gene encoding luciferase which can be assayed by a bioluminescence assay, the *uidA* gene encoding β -glucuronidase which can be assayed by a histochemical test, the *aphIV* gene encoding hygromycin phosphotransferase which can be assayed by testing for hygromycin resistance in the transformed organism, the *dhfr* gene encoding dihydrofolate reductase which can be assayed by testing for methotrexate resistance in the transformed organism, the *neo* gene encoding neomycin phosphotransferase which can be assayed by testing for kanamycin resistance in the transformed organism and the *lacZ* gene encoding β -galactosidase which can be assayed by a histochemical test. All of these reporter genes are obtainable from *E.coli* except for the *lux* gene. Sources of the *lux* gene include the luminescent bacteria *Vibrio harveyii* and *V.fischeri*, the firefly *Photinus pyralis* and the marine organism *Renilla reniformis*.

Alternatively, the first nucleic acid sequence may encode a protein of interest. The protein of interest may be a therapeutic protein including, but not limited to, a growth factor, differentiation factor, peptide hormone, enzyme e.g horse radish peroxidase (HRP) or alkaline phosphatase (ALP), protein kinase, structural protein, cytokine, antigen for example HLA antigen, protein components of the complement system, or any biologically active protein, for example, a receptor, immunoglobulin, or "reporter" protein or any biologically active fragment thereof. Preferably, the first nucleic acid sequence encodes a therapeutic protein which is soluble complement receptor I.

10 The DNA encoding the first nucleic acid sequence of the invention may be single or double stranded. Single stranded DNA may be the coding or sense strand, or it may be the non-coding or anti-sense strand. For therapeutic use, the first gene is in a form capable of being expressed in the subject to be treated.

15 The termination sequence of the first aspect of the invention may be a sequence of adenylate nucleotides which encode a polyadenylation signal. Typically, the polyadenylation signal is recognisable in the subject to be treated, such as, for example, the corresponding sequences from viruses such as, for human treatment, the SV40 virus. Other termination signals are well known in the art and may be used.

20 Preferably, the polyadenylation signal is a bidirectional terminator of RNA transcription which is located between the first and second gene of the expression vector. The termination signal may be the polyadenylation signal of the simian 40 virus (SV40), for example the SV40 late poly(A). Alternatively, the termination sequence may be the polyadenylation signal of bovine growth hormone which results in maximal expression when combined with a CMV promoter (Yew *et al. Human Gene Therapy* ; 8: 575-584 (1997)).

25 In addition the expression vector may comprise a further polyadenylation sequence, for example an SV40 early poly(A). Such a further poly (A) may be located upstream

of the first gene to reduce cryptic transcription which may have initiated within the vector thereby ensuring that basal gene expression from the vector is minimal.

5 The second nucleic acid sequence of the expression vector encodes reverse tetracycline transactivator (rtTA) and can be composed of the mutant Tet repressor, reverse Tet repressor (rTetR) fused to a VP16 moiety (Gossen *et al*, *Science* **268** 1766-1769 (1995)). Reverse transactivator (rtTA) requires effector molecules which are tetracycline or certain tetracycline derivatives for specific DNA binding. The effector may be chlortetracycline, oxytetracycline or anhydrotetracycline or doxycycline.

10 Preferably the effector is doxycycline (Dox). Typically, the concentration of Dox required in cells to activate rtTA binding to tet operator elements in the autoregulatory expression vector is greater than 10ng/ml, preferably between 10ng/ml and 1 μ g/ml, for example between 50ng/ml and 900ng/ml, 100ng/ml and 800ng/ml, 200ng/ml and 700ng/ml, 300ng/ml and 600ng/ml or 400ng/ml and 500ng/ml.

15 The effector molecule generally determines whether rtTA binds to the tet operator sequence. In the absence of an appropriate effector, rtTA binding to the tet operator sequence may not take place thereby preventing transcriptional activation of the first and/or second nucleic acid sequence. Contrastingly, in the presence of an appropriate effector, rtTA binding to the tet operator sequence may take place and transcription of the first and/or second nucleic acid sequence from the promoter sequence may occur.

20 The expression vector of the first aspect of the invention is preferably arranged such that the first tetP is in sense orientation and the second tetP is in antisense orientation. The direct opposition of the two regulatable promoters may facilitate their own downregulation as rtTA levels are reduced through autoregulation following effector removal.

25 The autoregulatory expression vector of the first aspect of the present invention preferably results in low basal levels of rtTA expression in the absence of an effector

and in the presence of an effector leads to increased levels of rtTA through positive feedback thereby autoregulating expression of the first nucleic acid sequence.

5 Preferably, in this system low level expression takes place in the absence of an effector such as Dox. Rapid "switching on" of gene expression generally takes place in the presence of the effector and gene expression is rapidly "switched off" following removal of the effector.

10 One embodiment of the first aspect of the invention provides a DNA molecule having a first inducible promoter sequence operably linked to a first gene sequence, a second inducible promoter sequence operably linked to a second gene sequence, a termination sequence operably linked to the first and second gene sequences; wherein the first promoter sequence in sense orientation and the second promoter sequence in antisense orientation provides co-transcription of the gene sequences when the promoter 15 sequences are inducibly transcribed.

20 A second aspect of the invention provides an expression system comprising the autoregulatory expression vector of the first aspect of the invention. The expression system may comprise one or more host cells.

25 Representative examples of appropriate hosts cells for the autoregulatory expression vector include bacterial cells, such as *streptococci*, *staphylococci*, *E.coli*, *streptomyces* and *Bacillus subtilis*; fungal cells, such as yeast cells, for example *Saccharomyce cerevisiae*, and *Aspergillus* cells; insect cells such as *Drosophila* S2 and *Spodoptera* Sf9 cells; animal cells such as CHO, COS, C127, 3T3, BHK, 293 and Bowes melanoma cells and other suitable human cells; and plant cells.

30 Preferably, the host cells of the expression system are mammalian cells in which a low basal activity of tetP is observed. The basal activity of tetP has previously been shown to be dependent upon the mammalian cell type in which it is employed, with high

basal expression of genes observed in several cell lines (Freundlieb *et al.*, *J Gene Med*, 1: 4-12 (1999)).

Introduction of an expression vector into the host cell can be effected by calcium 5 phosphate transfection, DEAE-dextran mediated transfection, microinjection, cationic lipid-mediated transfection, electroporation, transduction, scrape loading, ballistic introduction, infection of other methods. Such methods are described in many standard laboratory manuals, such as Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring 10 Harbor, N.Y. (1989).

Mature proteins can be expressed in host cells including mammalian cells such as CHO cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can be employed to produce such proteins using RNA's 15 derived from the expression vector of the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989).

20 In a third aspect, the invention provides a method of treatment of a patient such as a mammal, including human, comprising administering to a recipient the autoregulatory expression vector of the first aspect of the invention or the expression system of the second aspect of the invention.

25 As used herein the term "treatment" includes any regime that can benefit a human or non-human animal. The treatment may be in respect of an existing condition or disorder, or may be prophylactic (preventative treatment). The treatment may be of an inherited or acquired disease. The treatment may be of an acute or chronic condition. Preferably, the treatment is of a chronic condition with relapsing symptoms. The first 30 nucleic acid sequence of the present invention may encode a protein for use in the

treatment of a genetic disorder including, but not limited to, cystic fibrosis, cancer, haemophilia, X-linked SCID, Huntington's chorea, Addison's disease or Graves' disease. Other disorders, falling within the definition genetic disorders, are also contemplated by the present invention including, but not limited to, rheumatoid 5 arthritis, diabetes mellitus or diabetes insipidus, multiple sclerosis, atherosclerosis, Alzheimer's disease, Parkinson's disease, Crohn's disease or any inflammatory disease.

10 An autoregulatory expression vector according to the first aspect, or an expression system according to the second aspect, of the invention may be used therapeutically in the method of the invention by way of gene therapy.

15 The first and second nucleic acid sequences of the invention, for use in therapy, generally may be inserted into the vector using standard techniques so that it is operably linked to the first tetP sequence for expression. Promoters for expression *in situ* of the nucleic acid sequences should desirably be recognised in the subject to be treated.

20 Administration of the autoregulatory expression vector of the first aspect, or the expression system of the second aspect, may be direct to the target site by physical methods. Examples of these include topical administration of the 'naked' nucleic acid vector in an appropriate vehicle for example in solution in a pharmaceutically acceptable excipient such as phosphate buffered saline, or administration of the vector 25 by physical methods such as particle bombardment according to methods known in the art.

30 Other physical methods for administering the nucleic acid directly to the recipient include ultrasound, electrical stimulation, electroporation and microseeding. Further methods of administration include oral administration or administration through inhalation.

Particularly preferred is the microseeding mode of delivery which is a system for delivering genetic material into cells *in situ* in a patient. This method is described in US Patent No. 5,697,901.

5 Expression vectors according to the invention may also be administered by means of delivery vectors. These include viral delivery vectors, such as adenovirus or retrovirus delivery vectors known in the art.

10 Other non-viral delivery vectors include lipid delivery vectors, including liposome delivery vehicles known in the art.

15 Administration may also take place via transformed host cells. Such cells include cells harvested from the subject, into which the nucleic acid is transferred by gene transfer methods known in the art, followed by growth of the transformed cells in culture and grafting to the subject.

20 As used herein the term "gene therapy" refers to the correction of disease-causing genes by recombinant genetic engineering of body cells (somatic gene therapy). Furthermore, gene therapy can be divided into *ex vivo* and *in vivo* techniques. *Ex vivo* gene therapy relates to the removal of body cells from a patient, treatment of the removed cells with a vector ie, a recombinant vector, and subsequent return of the treated cells to the patient. *In vivo* gene therapy relates to the direct administration of the recombinant gene vector by, for example, intravenous or intramuscular injection.

25 Preferably the method of gene therapy of the present invention is carried out *ex vivo*.

Preferably in gene therapy, the expression vector of the present invention is administered such that it is expressed in the subject to be treated. Thus for human gene

therapy, the promoter is preferably a human promoter from a human gene, or from a gene which is typically expressed in humans, such as the promoter from human CMV.

5 For gene therapy, the present invention may provide a method for manipulating the somatic cells of human and non-human mammals.

10 The present invention therefore provides a method for providing a human with a therapeutic protein comprising introducing mammalian cells into a human, the human cells having been treated *in vitro* to insert therein an autoregulatory expression vector, according to the first aspect of the invention, encoding a therapeutic protein, the human cells expressing *in vivo* in the human a therapeutically effective amount of the therapeutic protein.

15 Each of the individual steps of the *ex vivo* somatic gene therapy method are also covered by the present invention. For example, the step of manipulating the cells removed from a patient with the expression vector or expression system of the present invention. As used herein, the term "manipulated cells" covers cells transfected with a recombinant vector.

20 Also contemplated is the use of the transfected cells in the manufacture of a medicament for the treatment of a genetic disorder.

25 A fourth aspect of the invention provides products containing an expression vector according to the first aspect of the invention and a further expression vector comprising a second DNA molecule as a combined preparation for simultaneous, separate or sequential use in gene therapy. Preferably the second DNA molecule is a nucleic acid vector comprising a nucleic acid sequence encoding a protein of interest as described herein. The nucleic acid sequence may be linked to a promoter-tet operator sequence (tetP) and a termination sequence. The promoter may be any suitable promoter, for example the human cytomegalovirus (CMV) promoter.

Preferably, the promoter comprises the minimum sequence required for promoter activity, for example, mCMV.

The nucleic acid sequence may encode a protein which is a "reporter" protein. In this
5 the second DNA molecule can be used as an accurate marker of a functional autoregulatory expression vector according to the first aspect of the invention. Alternatively, the nucleic acid sequence may encode a protein which is a therapeutic protein as described herein.

10 Typically the second DNA molecule is a bacterial plasmid, the induction of which is dependent upon rtTA expression from the autoregulatory expression vector of the first aspect of the invention or is co-expressed using an internal ribosome entry site (IRES) which may be located after the stop codon of the first DNA molecule and the initiation codon of the second DNA molecule.

15 Co-regulating the expression of two nucleic acid sequences which both encode therapeutic proteins may be achieved by administration of the products of the fourth aspect wherein the second DNA molecule encodes a therapeutic protein. Co-regulated expression of two such nucleic acid sequences has wide potential application in many
20 therapeutic situations, but will be of optimum benefit where the gene products have additive or synergistic therapeutic effect.

Preferred features of the second and subsequent aspects of the invention are as for the first aspect *mutadis mutandis*.

25 All preferred features of the first, second and third aspects of the present invention apply, *mutadis mutandis*, to the fourth and subsequent aspects of the invention.

30 There is a requirement for expression systems which have an application in gene therapy for the treatment of chronic conditions with relapsing symptoms. In particular,

there is a need for a regulatory system in which expression of a therapeutic protein is restricted to periods when relapses occur to prevent potential side effects from long term expression of therapeutic proteins.

5 The autoregulatory expression vector of the present invention demonstrates regulatory properties which allow for a greater degree of control over the expression of a nucleic acid encoding a therapeutic protein ensuring that cells are not exposed to therapeutic proteins for a prolonged period of time.

10 Thus, a fifth aspect of the invention provides an autoregulatory expression vector according to the first aspect of the invention or an expression system according to the second aspect for use in medicine, preferably for use in gene therapy.

15 A sixth aspect of the invention provides for the use of an autoregulatory expression vector according to the first aspect of the invention or an expression system according to the second aspect of the invention in the manufacture of a medicament for the treatment of a genetic disorder.

20 The invention also relates to compositions comprising the autoregulatory expression vector according to the first aspect of the invention or the expression system according to the second aspect of the invention. Therefore, the expression vector or expression system of the present invention may be employed in combination with a pharmaceutically acceptable carrier or carriers.

25 Such carriers may include, but are not limited to, saline, buffered saline, dextrose, liposomes, water, glycerol, ethanol and combinations thereof.

30 The autoregulatory expression vector or expression system of the present invention may be employed alone or in conjunction with other compounds, such as therapeutic compounds.

5 The pharmaceutical compositions may be administered in any effective, convenient manner effective for treating a patients disease including, for instance, administration by oral, topical, intravenous, intramuscular, intranasal, or intradermal routes among others. In therapy or as a prophylactic, the active agent may be administered to an individual as an injectable composition, for example as a sterile aqueous dispersion, preferably isotonic.

10 For administration to mammals, and particularly humans, it is expected that the daily dosage of the active agent will be from 0.01mg/kg body weight, typically around 1mg/kg. The physician in any event will determine the actual dosage which will be most suitable for an individual which will be dependant on factors including the age, weight and response of the individual. The above dosages are exemplary of the average case. There can, of course, be instances where higher or lower dosages are
15 merited, and such are within the scope of this invention

20 The invention also provides a kit of parts comprising an autoregulatory expression vector according to the first aspect of the invention or an expression system according to the second aspect of the invention and an administration vehicle including, but not limited to, tablets for oral administration, inhalers for lung administration and
25 injectable solutions for intravenous administration.

25 The invention is now described by reference to the accompanying drawings;

30 FIGURE 1 Schematic diagrams of plasmid (A) and retroviral (B) vectors used in this study. tetP: seven repeats of tetracycline operator driving a minimal CMV promotor; EGFP: enhanced green fluorescent protein; CMV IE: CMV immediate-early enhancer/promotor; rtTA: reverse tetracycline transactivator; LTR: long terminal repeat; sCR1:

truncated soluble complement receptor 1; SV40 early/late poly(A) signal (solid triangle); β -globin poly(A) signal (open triangle); downstream SV40 untranslated region (grey box). Numbers in parenthesis represent the length of each vector in base pairs.

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FIGURE 2 Comparative expression of luciferase from self-contained retroviral and plasmid vectors. DTF were plated at 5×10^5 cells on 6 cm plates and were transiently transfected with equivalent molar amounts of DNA to 30 μ g of pGRTL. Luciferase expression was induced with Dox (1 μ g/ml) for 48 hours, and measured values were adjusted for protein content. Each value is the mean from duplicate transfections.

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FIGURE 3 (A) Luciferase expression from PGL2 basic, pGTL and pGRTL in the absence of Dox was assessed 24 hours after transient transfection of DTF (5×10^5 cells/well on a 6 well plate) with 10 μ g pGL2 basic and equivalent molar amounts of pGTL and pGRTL. Each value is the mean luciferase measurement adjusted for protein content from triplicate transfections and vertical bars represent SEM. (B) Luciferase expression from pGRTL in the presence of Dox (1 μ g/ml) was assessed at 6, 24 and 48 hours after transient transfection of DTF (2.5×10^5 cells/well on a 6 well plate) and compared at the same time points to pGCMV (5 μ g) from which luciferase is constitutively expressed. Luciferase activity is expressed as percentage of pGCMV values.

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FIGURE 4 (A) Induction of luciferase expression from a selection of clones for 24 hours with Dox (1 μ g/ml). Numbers above the bars indicated the fold induction. (B) Luciferase expression from DTF-GRTL clone 20. Cells were plated at 2.5×10^5 /well and were induced with Dox (1 μ g/ml) for 72 hours. Values are the mean luciferase measurement adjusted for

protein content from triplicate repeats. Vertical bars represent SEM, * indicates a significance of $p<0.005$ between non-induced and induced values. (C) The kinetics of “on” and “off” luciferase expression from DTF-GTRTL clone 10. Luciferase expression following both switching “on” (white circles) and “off” (black circles) at time points 24, 48 and 72 hours was determined as the percentage change between 0% - non-induced cells, and 100% - cells continuously induced throughout the course of the experiment.

10 FIGURE 5 (A) Induction of sCR1 from DTF permanently transfected with pGTRTC. Each point is the mean of triplicate values and vertical bars represent SEM (p values are indicated as * $p<0.05$, ** $p<0.01$ and *** $p<0.005$ for significant differences between non-induced and induced values). Numbers above columns indicate the magnitude of induction in the presence of DOX. The symbol ~ indicates that the fold induction could not be calculated because the non-induced sCR1 level was below the detection limits of the ELISA. (B) Kinetics of “on” and “off” sCR1 expression from CTRTC clone 1. SCR1 values are shown on a long scale as the mean from triplicate repeats and vertical bars represent the SEM. Significant differences between switched “on” and non-induced cells and between switched “off” and continuously induced cells of $p<0.05$, $p<0.01$ and $p<0.005$ are indicated by *, ** and *** respectively.

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25 FIGURE 6 A: EGFP expression detected by fluorescence microscopy and attributed a score from 0-3 in accordance with the criteria outlined in the Examples. B: sCR1 expression determined by SDS-PAGE electrophoresis and detected on Western blots (see inset image) was quantitated by densitometric volume analysis. Numbers above columns

indicated the magnitude of sCR1 induction in the presence of Dox on the basis of densitometry measurements.

FIGURE 7 Induction of sCR1 and EGFP in DTF-GTRTC+GTE Clone 21. (A) 5 sCR1 levels. (B) fluorescence values determined as the median value for the population. Each Dox induced value is the mean of triplicate values, vertical lines represent SEM and *, ** and *** represent significance values of $p<0.05$, $p<0.01$ and $p<0.005$ respectively compared with the average non-induced value.

FIGURE 8 Luciferase expression from Cos 7 cells transfected with pGTL alone or 10 in combination with pUHG 17.1, or expression Vectors for GATA 4, GATA 5 and GATA 6 at a ratio of 1:1. Cells transfected with pUHG 15 17.1 were cultured in the absence or presence of Dox (1 μ g/ml). Luciferase levels were determined 24 hours after transfection. Values are the mean of triplicates and vertical bars represent SEM. * indicates significant difference from the pGTL transaction of $p<0.05$, whilst ^ represents a p value of 0.053 between the GTL and GTL + GATA 5 groups.

FIGURE 9 Luciferase expression from Cos 7 cells transfected with PGTL alone or 20 in combination with pUHG 17.1, and the expression vectors for GATA 4, GATA 5 and GATA 6 at a ratio of 1:1:1. Cells transfected with pUHG 25 17.1 were cultured in the absence or presence of Dox (1 μ g/ml) and all transfections with GATA factors were induced with Dox. Luciferase levels were determined 24 hours after transfection. Values are the mean of triplicates and vertical bars represent SEM. * and ** indicate significant difference from the pGTL and pUHG 17.1 with Dox transfection of $p<0.05$ and $p<0.01$ respectively.

FIGURE 10 Luciferase expression from DTF transfected cells with GTRTL (5 μ g) and pcEGFP (inert plasmid) or with ApHRO (retroviral plasmid encoding mIFN β). Transfected cells were cultured in the absence or presence of Dox for 48 hours. Values are the mean of triplicates and vertical bars represent SEM. Values for fold induction are indicated above columns and are calculated by comparison to the pGTRTL + pcEGFP – Dox sample. * indicates a significant ($p<0.005$.) difference from the pGTRTL + pcEGFP – Dox value, and \$ indicates a significant ($p<0.05$.) difference from the pGTRTL + pcEGFP + Dox value.

FIGURE 11 Schematic representation demonstrating the position and orientation of the SV40 poly A signal in the plasmids studied. The symbol $\rightarrow\leftarrow$ indicates the direction of early (E) and late (L) SV40 polyA signals. TetP – tetracycline responsive promoter; rtTA – reverse tetracycline transactivator; Luc/Luc+ - luciferase gene; 3'UTR – 3' untranslated region.

FIGURE 12 Luciferase expression from NB100 cells transfected with pGTRTL and pGTRTL* and cultured in the absence and presence of Dox (1 μ g/ml). Values are the mean of triplicate transfections and vertical bars represent SEM. Numbers above bars indicates the fold induction, whilst significant induction with Dox above non-induced levels is indicated by * ($p<0.005$) and ** ($P<0.001$). Significantly higher luciferase values of pGTRTL are indicated by \$ ($p<0.01$) and \$\$ ($p<0.0005$).

The invention is now described with reference to the following, non-limiting examples;

Example 1- Vector preparation and cloningCells

DBA tst fibroblast (DTF) are primary embryonic fibroblasts immortalised with the temperature sensitive T antigen were grown in DMEM supplemented with 10% FCS, 5 penicillin (100 U/ml), streptomycin (100 µg/ml) and 2 mM glutamine as previously described (Triantaphyllopoulos *et al*, *Gene Therapy* 5 253-263 (1998)). The retroviral packaging cell line GP+E86 was maintained in the same supplemented DMEM.

Cloning Strategy

10 The plasmids pUHG17-1, pUHD10-3 and pUHC13-3 were generously provided by Gossen and Bujard (Universitat Heidelberg, Germany).

Retroviral constructs were based on a previously described mini MFG murine Moloney retroviral vector (Neve *et al*, *Cytokine* 8 365-370 (1996)). The 15 cytomegalovirus immediate/early promoter-rtTA cassette (CMV-rtTA) was removed from pUHG17-1 as an Xho 1-blunt-Bam H1 fragment and cloned into mini MFG restricted with Bst X1-blunt-Bam H1 resulting in the vector MFGCMVrtTA.

20 The seven repeats of the tetracycline operon located upstream from a minimal CMV promoter (tetP) was removed from pUHD10-3 by Xho 1-Eco R1 digest, blunted and subcloned into pBluescript II KS (Stratagene Inc. La Jolla, CA, USA) which had been restricted with Asp 718-Xho 1 and also blunted with klenow, producing the vector pBStet.

25 The tetP cassette with the downstream multiple cloning site (MCS) was removed from pBStet by restriction with Xho 1-Xba 1, blunted and ligated with Bgl II linkers, restricted with Bgl II and cloned into MFGCMVrtTA cut with Bam H1. Two vectors were obtained, MGTT-1 and MGTT-2 in which the tetP is located downstream of the rtTA in the sense and anti-sense direction respectively.

5 Luciferase was cloned into MGTT-1 and MGTT-2 from pGL2 basic (Promega Corp., Madison, USA). Luciferase along with a downstream SV40 untranslated region and poly(A) were removed from pGL2 basic by restriction with Bam H1 and Bgl II, and cloned into the retroviral vectors linearised with Bam H1, the resulting vectors were called M1-LUC-CMV and M2-LUC-CMV (Figure 1).

10 The self-contained inducible plasmids and intermediary subclones were all constructed on the pGL2 basic backbone. The restriction site Bbs I in the mCMV is common to both the full-length CMV I/E and the tetP promoter and was utilised in constructing the autoregulatory rtTA unit.

15 The plasmid pUHC13-3 was restricted with Xho 1 and Bbs I and the released fragment was inserted upstream of rtTA in pUHG17-1 cut Xho I – Bbs I, crating plasmid pRT. The CMV-rtTA and tetP-rtTA cassettes were removed by restriction with Xho I –
20 Bam HI from pUHG17-1 and pRT respectively and inserted in the 3'-5' orientation in the Sal-1 – BamH1 sites of pGL2 basic, the resulting vectors were named pGR and pGTR. The plasmid pUHC13-3 was subsequently cut 5' of the tetP with Xho I and towards the 3' terminus of luciferase with Cla I. This cassette was cloned into both pGR and pGTR cut Xho I – Cla I, thus reconstituting the full-length luciferase gene. The constructs obtained were termed pGRTL and pGTRTL respectively (Figure 1).

25 The inducible luciferase plasmid pGTL equivalent to pUHC13-3 but on the pGL2 backbone was constructed by restricting pGL2 basic with Xho I – Cla I, and ligating in the tetP-luciferase cassette cut with the same enzymes from pUHC 13-3.

20 The plasmid pGT with a tetP upstream from a MCS on the pGL2 basic backbone is equivalent to pUHC 10-3 and was cloned by removing the tetP-MCS cassette from pBStet with Bst XI – blunted – Xho I and inserting it into pGTL with the tetP-luciferase cassette removed by restriction with Xho I – Eco RV.

An inducible vector pGTEGFP expressing enhanced green fluorescent protein (EGFP) was also constructed by removal of the EGFP gene from pEGFP-1 (Clontech, Palo Alto, California, USA) by Eco RI – Not I restriction and inserted into pGT cut with the same enzymes (Figure 1).

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The retroviral vector MFG-CR1, which expresses a truncated form of sCR1, was used to obtain the sCR1 gene. The sCR1 gene was removed from this vector by the restriction Nco 1 – blunted – Not I, and cloned into the pGT vector cut with Eco RV – Not I, forming the inducible vector pGTC1. The autoregulatory vector expressing sCR1 required transfer of the tetP-sCR1 cassette (restricted Nhe I – Bsp MI) from pGTC1 and cloned into pGTRTL cut with the same enzymes, producing the autoregulatory vector pGTRTC (Figure 1).

Preparation of DNA

15 Large-scale preparation of DNA was performed by the standard caesium chloride method (Sambrook *et al*, Molecular Cloning: A Laboratory Manual, *Cold Spring Harbor Laboratory Press, USA*, second edition: (1989)). Purified DNA was resuspended in sterilised distilled water and the DNA concentration was determined by absorbance measurement at 260nm.

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Example 2 – Cell transfection studies

Methods

Transient Transfection

25 Transient transfection was used to compare the induction characteristics of self-contained vectors. DTF were seeded in 6 well plates at $0.25-0.5 \times 10^6$ cells per well. The next day cells were transfected with plasmids using molar equivalents of DNA in order to ensure accurate comparison of plasmid expression. DNA was precipitated was calcium phosphate for 20 minutes before addition to cells and incubated for 30 minutes at room temperature, then 4 ml medium was added and incubated overnight at

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37°C/10% CO₂. The next day an osmotic shock was performed. Briefly, medium was removed from the plate and 500 µl of 10% glycerol in serum-free medium was gently added to the plate and incubated with the cells for 4 minutes with gentle tilting of the plate. Cells were then washed twice with 3 ml serum free medium, and then cells were cultured overnight in 3 ml medium with or without Dox (1 µg/ml). Experiments were terminated between 24 and 72 hours later when protein expression was determined in either the cell lysates or supernatants.

Permanent Transfection

Cells for transfection were seeded on 9 cm plates at 0.5x10⁶ cells per plate. The next day cells were co-transfected with either an inducible retroviral vector (20 µg) and the selection construct pSV2Neo (1 µg) (Southern *et al*, *J. Mol. Appl. Genet.* **1** 327-341 (1982)) or an inducible plasmid vector (20 µg) and the selection construct pTK-Hyg (Clontech, Palo Alto, California, USA) (1 µg).

Inducible plasmid vectors, as described in Example 1, were either transfected in isolation as 20 µg of a single autoregulatory vector (i.e. pGTRTL) or in combination with 10 µg each of an autoregulatory and inducible vector (i.e. pGTRTC and pGTEGFP). The DNA was precipitated as above and cells treated with 10% glycerol. The next day cells were split 1 to 5 and cultured overnight in normal medium for a further 24 hours before selection was initiated by growth in normal medium supplemented with the respective selection antibiotic, G418 (1 mg/ml) (Life Technologies Inc, Paisley, Scotland) or hygromycin B (200 µg/ml) (Calbiochem-Novabiochem Corp, La Jolla, CA, USA). The selection medium was changed twice weekly and single clones were visible after three weeks.

Clones were isolated by washing plates with PBS and placing discs of autoclaved 3MM paper soaked in trypsin/EDTA over visible clones. Plates were returned to the incubator for 4 minutes after which the paper was transferred to individual wells in 12 well plates containing 1 ml Hygromycin selection medium. Clones were grown until

approximately 70% confluence before examining Dox inducible expression of sCR1, or were split to triplicate wells for examination of luciferase expression. Kinetics of gene regulation of stable clones were performed by plating cells in 6 or 12 well plates at 1×10^5 – 2.5×10^5 cells per well and gene expression was assessed at time points from 5 6 hours up to 72 hours.

The kinetics of “on” and “off” luciferase expression from DTF-GTRTL clone 10 was assessed. Prior to plating, cells were grown in media with or without Dox (1 $\mu\text{g}/\text{ml}$ for 72 hours. Cells were then replated at 2.5×10^5 /well on 6 well plates in the same culture 10 mediate. The next day, 3 wells of induced and non-induced cells were switched to the opposite media for 72 hours. On the following two days 3 well of induced and non-induced cells were switched to the opposite media for 48 and 24 hours respectively. Luciferase expression was measured as described below.

15 Induction of sCR1 from DTF permanently transfected with pGTRTC was assessed. Clones isolated by selection in hygromycin media were plated at 2.5×10^5 cells/well on a 6 well plate. The next day cells in 3 wells were induced with Dox (1 $\mu\text{g}/\text{ml}$) containing media and non-induced cells were maintained in normal media (3 ml). The sCR1 levels in the media after 48 hours were measured by ELISA (see below).

20 The kinetics of “on” and “off” sCR1 expression from CTRTC clone 1 was assessed. The experiment was performed in the same way as luciferase “on”/“off” expression kinetics described above except this experiment was performed on a 12 well plate with induced and non-induced cells plated at 1×10^5 cells/well. In addition sCR1 is secreted 25 from cells, so media was removed at 24 hour intervals for measurement of sCR1 and was replaced with an equal volume of fresh media with or without Dox.

30 Induction of sCR1 and EGFP in DTF-GTRTC+GTE Clone 21 was assessed. Cells were plated in 1 ml normal media at 1×10^5 /well in 12 well plates. The next day cells in 3 wells were induced with Dox (1 $\mu\text{g}/\text{ml}$) containing media for 72 hours, and this

was repeated for triplicate wells on the subsequent two days for 48 and 24 hours induction. sCR1 levels in the media were measured by ELISA (see below).

Luciferase Assay

5 Luciferase activity in cell lysates was measured by lysing cells on 6 or 12 well plates at room temperature in 200 µl of lysis buffer (25 mM Tris-phosphate (pH 7.8), 2 mM Dithiothreitol (DTT), 2 mM 1,2 diaminocyclohexane-N,N,N',-N'-tetraacetic acid, 10% glycerol, 1% triton X-100). Insoluble material was pelleted by centrifugation for 1 minute at 13,000 rpm, 20 µl of the supernatant was mixed with 100 µl of luciferin reagent (20 mM Tris, 1.07 mM (MgCO₃)₄Mg(OH)₂.5H₂O, MgSO₄, 0.1mM EDTA, 33.3mM DTT 270 µM Coenzyme A, 470 µM luciferin, 530 µM ATP) and the light produced in 10 seconds was measured in a luminometer (either a Luminoskan, Labsystems, Helsinki, Finland, or a MLX Microtiter® Plate Luminometer, Dynex Technologies Inc., Chantilly, Virginia, USA).

10 15 Luciferase values were normalised by measuring the total protein concentration in cell lysates using the Bradford protein assay (Bio-Rad Laboratories Inc., Hercules, California USA) and values of luciferase activity were expressed as relative light units (RLU) per µg of protein.

20 25 Measurement of sCR1 by Western Blotting and ELISA

Western Blotting: Clones were cultured until they reached 70% confluence, then the selection medium was removed and replaced by 1 ml serum free selection medium. Cells were cultured for 24 hours and then the supernatant was collected and replaced by 1.0 ml serum free selection medium containing 1 µg/ml Dox. Again incubation was continued for 24 hours and supernatants were concentrated 10-fold for measurement of sCR1 by Western blot. SDS was added to the supernatants at a final concentration of 0.5%, and boiled for 5 minutes before addition of methanol (10 ml) and protein precipitated overnight at -20°C. The following day the precipitate was pelleted by centrifugation at 3000 rpm/15 minutes. The supernatant was discarded and

the pellet left to dry at room temperature for 10 minutes. The pellet was resuspended in 50 µl PBS and 50 µl 2xSDS (Laemmli, *Nature* 227 680-685 (1970)) loading buffer and then boiled for 10 minutes.

5 Concentrated sCR1 samples (30 µl) were run on SDS-PAGE (Laemmli, *Nature* 227 680-685 (1970)) and transferred to nitrocellulose (Schleicher and Schuell, Dassel, Germany). Blots were blocked with a 5% non fat dry milk in TBS (20 mM Tris-HCl, 200 mM NaCl, 0.1% Tween 20, pH 7.5) for 2 hours at room temperature, before detection of sCR1 by incubation with mAb YZ1 (gift from Prof. D. Fearon, 10 Cambridge University) diluted 1:1000 with blocking solution for 1 hour. Blots were then washed 3 x 30 minutes with high-salt TBS (500 mM NaCl), and then incubated with biotinylated anti-mouse IgG (Amersham International plc, Buckingham, UK) diluted 1:2000. Blots were again washed 3 x 15 minutes with PBS supplemented with 0.01% Tween 20. Finally blots were incubated with ECL reagent (Amersham 15 International plc) for 1 minute and then wrapped in Saran wrap before exposure to photographic Hyperfilm™-MP (Amersham) to visualise detected sCR1.

20 Semi-quantitative measurement of sCR1 was performed by densitometric measurement using an imaging densitometer (Model GS-670, Bio-Rad).

25 *ELISA*: The direct measurement of sCR1 in normal media by ELISA was developed by one of us (H.D) as a standard procedure during the course of this study. ELISA plates were coated overnight at 4°C with anti-CR1 mAb J3.D3 (Serotec Ltd, Kidlington, Oxford, UK) diluted 1:2000 with PBS. The following day plates were blocked with a 1% bovine serum albumin (Sigma) solution in PBS for 2 hours.

Standards of full length sCR1 (gift from Prof. D. Fearon, Cambridge University) (in the range 5 µg/ml to 160 pg/ml) and samples were then loaded on washed plates and incubated for 3 hours. Plates were again washed and sCR1 detected with a polyclonal

rabbit antisera (gift from Dr. R. Smith, AdProtec plc, Royston, Hertfordshire, UK) diluted 1:2000 with PBS for 1 hour.

Second layer detection was performed with anti-rabbit Ig, horseradish-peroxidase linked F(ab)₂ fragment from donkey (Amersham) which was diluted 1:2000 with PBS and incubated for 1 hour. Signal was detected using the TMB microwell substrate system (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD, USA) and the reaction stopped by addition of 1M phosphoric acid. Absorbance measurements were performed at 450 nm using an EL 312e microplate biokinetics reader (Bio-Tek Instruments, Inc. CA, USA).

Quantitation of EGFP Expression by Cell Fluorescence

Fluorescent cells were visualised using an inverted UV microscope (Fluovert, Leitz, Germany). For a semi-quantitative assessment of cell fluorescence clones were attributed a score from 0 to 3 (0, no fluorescence; 1, background or low fluorescence; 2, medium fluorescence; and 3, high fluorescence). Fluorescence was accurately quantitated with cells fixed with PBS containing 2% paraformaldehyde by flow cytometry using FACScan II (Beckton Dickinson, Mount View, CA), and fluorescence data was analysed using Cell Quest software.

Semi quantitative expression of EGFP and sCR1 from DTF clones co-transfected with pGTRTC and pGTE and selected in hygromycin supplemented media was assessed. Clones were plated in single wells on 12 well plates in 1 ml of serum-free media. After 24 hours non-induced fluorescence of cells was assessed as described above. Media was collected for sCR1 measurement and replaced with 1 ml of serum-free media containing Dox (1 µg/ml). Fluorescence was reassessed in media collected after 24 hours Dox induction.

Statistical Methods

Descriptive statistics, correlation and Students t Test for two sample data of unequal variance were performed using Microsoft® Excel 98 software.

5

ResultsComparative Induction Characteristics of Retroviral and Plasmid Vectors

Preliminary transfection studies confirmed that DTF are a suitable cell line for the study of tetracycline inducible expression. Co-transfection of pUHC 13-3 and pUHG 10 17-1 in a ratio between 1:10 and 1:1 was optimal for Dox regulated luciferase expression from DTF by transient transfection.

Comparative induction of luciferase from retroviral (M1 LUC CMV and M2 LUC CMV) and plasmid constructs (pGRTL and pGTRTL) was performed by transient transfection of DTF. Following induction with Dox for 48 hours the degree of gene induction was 23, 21, 12 and 34-fold respectively (Figure 2). In terms of the induced 15 level of luciferase expression the ranked order for these vectors is pGTRTL > M2 LUC CMV > M1 LUC CMV = pGRTL. For quantitative analysis of the level of luciferase expression, these vectors were compared to the constitutive CMV promoter 20 in the construct pGCMV by transient transfection of DTF with equivalent amounts of each vector. After 24 hours induction with Dox, luciferase expression from pGTRTL was equivalent to 25% of the CMV IE while expression from pGRTL was less than 1% (Table 1).

25 Comparison of Basal and Induced Luciferase Expression from GTRTL to Expression from pGTL and pGCMV

The basal activity of the tetP in DTF was assessed by comparison of luciferase expression following transient transfection with pGTL and pGL2 basic. Luciferase expression was significantly increased ($p<0.0005$) in excess of a 1000-fold from the 30 tetP containing plasmid pGTL compared with the promoterless vector pGL2 basic

(Figure 3a). The basal level of luciferase expression from pGRTL was also compared to pGTL, both plasmids are identical except for the inclusion of the tetP-rtTA cassette in pGRTL.

5 Results in Figure 3a illustrate that in the absence of Dox, luciferase expression from pGRTL was higher at 119.9 RLU compared to 55.23 RLU for pGTL, indicating a slight but significant ($p<0.05$) increase in promoter activity by inclusion of the tetP-rtTA cassette in pGRTL.

10 Comparison of Dox induction from pGRTL with constitutive expression from pGCMV over a period of 48 hours revealed that luciferase expression, as a percentage of CMV driven expression, increased to 4%, 38% and 81% at intervals of 6, 24 and 48 hours (Figure 3b). Basal expression of luciferase from pGRTL was in the region of 2-3% of the constitutive CMV promoter throughout the 48 hour time course.

15 Stable Transfections

DTF were permanently transfected with pGRTL by co-transfection with the enhancerless selection vector pTKHyg at a ratio of 20:1. Following selection with hygromycin B, 28 clones were obtained for pGRTL, of these, 20 displayed inducible luciferase expression in response to Dox induction for 24 hours. Luciferase expression from the remaining 8 clones was low with expression from non-induced cells below detection limits. The induction of luciferase from a selection of these clones is illustrated in Figure 4a. Both the degree of induction and level of luciferase expression varied widely between the clones.

25 Twelve stable clones were obtained for pGRTL, of these only one was responsive to Dox induction, and again the level of luciferase expression observed was low (not shown). Retroviral constructs M1 LUC CMV and M2 LUC CMV were stably transfected in to the GP+E86 packaging cells by co-transfection with the selection plasmid pSV2Neo. No stable clones were obtained for M1 LUC CMV, and the best

stable clone for M2 LUC CMV displayed Dox regulated expression of luciferase up to 25-fold (not shown).

Kinetics of Luciferase Regulation from a Stable Clone

5 The highest degree of luciferase regulation was observed with clone 21, which displayed an increase of 62-fold after 72 hours Dox induction (Figure 4b).

10 DTF-GTRTL Clone 10 was used as a representative clone to examine the 'On'/'off' kinetics of luciferase expression at time points of 24, 48 and 72 hours after switching maximally induced cells (induced with Dox for 96 hours) to Dox free media, and non-induced cells to Dox containing media. Luciferase expression was shown to be rapidly up and down regulated following the addition or removal of Dox respectively. Induced cells started to plateau after 48 hours (Figure 4c) whilst expression was rapidly switched 'off' following removal of Dox reaching 8% above baseline after 48 15 hours (Figure 4c).

Stable Transfectants with GTRTC

20 Stable DTF clones expressing sCR1 were obtained after transfection with pGTRTC (20 µg) with pTKHyg (1 µg) and selected in hygromycin B containing medium. Thirty one clones were tested for inducible Dox expression of sCR1, of these 30 25 demonstrated inducible sCR1 expression detected by Western blot after 24 hours induction (not shown). A selection of these clones were induced with Dox for 48 hours and sCR1 expression determined by ELISA (Figure 5a). Induction of sCR1 was significant for four of these clones. As with stable pGTRTL clones a range of induction levels and expression levels were observed, with clone 2 displaying the greatest degree of induction at 74-fold and clone 4 achieving the highest induced level of expression.

'On'/'Off' Regulation of sCR1 Expression from a Stable DTF GTRTC Clone

'On' and 'off' kinetics of sCR1 expression was examined in DTF GTRTC clone 1 at 24 hour intervals over a 72 hour time course. Before examining the 'on'-'off' kinetics of sCR1 expression, non-induced cells were maintained in Dox free media, and induced cells were grown in Dox (1 µg/ml) containing media for 96 hours. Induction of sCR1 expression was assessed as the expression of sCR1 in a 24 hour period following Dox induction of non-induced cells. The switching 'off' of sCR1 expression was determined as the level of sCR1 expression in a 24 hour period after switching induced cells to Dox free media.

10 Levels of sCR1 were significantly increased by Dox induction at 24 and 48 hours compared to non-induced levels with, expression after 48 hours equivalent to continuously induced cells. The degree of sCR1 induction was 10, 42 and 54.5 fold at 24, 48 and 72 hours respectively (Figure 5b). Culture of maximally induced cells in 15 Dox free media resulted in a rapid reduction in sCR1 expression which was significantly lower than continuously induced cells at 24, 48 and 72 hours. Complete switching 'off' of sCR1 expression was observed after 48 hours. The reduction in sCR1 expression reflected a decrease of 4.5, 35 and 74 fold compared with continuously induced cells after 24, 48 and 72 hours respectively (Figure 5b). There is 20 a background increase in sCR1 expression for all groups due to the increase in cell numbers over the course of the experiment.

Dual Regulation of sCR1 and EGFP in a Single Cell

Regulated expression of both sCR1 and EGFP was examined in DTF clones obtained 25 after co-transfection with pGTRTC (10 µg), pGTE (10 µg) and pTKHyg (1 µg) and selection for hygromycin B resistance. The use of an inducible GTE construct in this transfection should act as a marker for clones containing pGTRTC as Dox induction of EGFP is dependent upon rtTA expression from the autoregulatory plasmid pGTRTC. Eight clones were induced with Dox for 48 hours. After induction 5 clones

were observed to express EGFP by fluorescence microscopy and were attributed a score from 1-3 (Figure 6a).

5 Expression of sCR1 was detected by Western blot for the same clones, and the expression pattern matched that of EGFP (Figure 6b). These results confirm that an inducible EGFP plasmid can be used as an accurate marker of a functional autoregulatory vector. The co-expression of EGFP and sCR1 was examined more closely with clone 21.

10 Expression of sCR1 and EGFP from clone 21 was monitored at time points of 24, 48 and 72 hours after induction with Dox. Expression of sCR1 measured by ELISA, showed significant increase over the course of the experiment to give maximum induction of 51 fold after 72 hours (Figure 7a). Expression of EGFP in the same cells was measured by flow cytometry and also showed similar kinetics of expression with 15 a maximum induction of 16 fold observed after 72 hours (Figure 7b). The parallel expression of sCR1 and EGFP in these cells shows good correlation over the 72 hour time course at $r=0.837$.

Conclusions

20 Comparative regulated expression of the reporter gene luciferase from these vectors in a transient assay indicated that the degree of regulation and the induced level of luciferase expression were markedly lower from the self-contained retroviral vectors M1 LUC CMV, M2 LUC CMV and the plasmid pGRT1 which contain the rtTA driven by the CMV IE promoter as compared with the enhancerless autoregulatory self-contained plasmid pGTRTL where both luciferase and rtTA are expressed from tetP 25 promoters. This difference indicates that the CMV IE in some way compromises the regulatory capacity of the tetP promoter. Despite the close structural similarity the induced expression of luciferase after 24 hours induction was considerably different at 7.69% and 0.87% relative to expression from a CMV IE promoter.

Expression of luciferase from pGTL provides a measure of the innate promoter activity of the tetP. The tetP displays significant promoter activity by comparison to the promoterless plasmid pGL2 basic. The basal activity of tetP has previously been shown to be dependent upon the cell type in which it is employed, with high basal expression of genes observed in several cell lines (Freundlieb *et al.*, *J Gene Med*; **1**: 4-12 (1999)). With the exception of the tetP-rtTA cassette the plasmid pGTL is identical to pGTRTL as both plasmids are constructed on the pGL2 basic backbone. The small increase in basal luciferase expression from pGTRTL causes an additional increase in promoter activity in the non-induced states.

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The strength of the induced promoter activity in pGTRTL was shown to approach that of a constitutive CMV IE promoter which is the strongest known constitutive promoter in most eukaryotic cells (Foecking & *Gene*; **45**: 101-105 (1986)).

15

The degree of regulated luciferase expression from pGTRTL was in the order of 30 fold in transiently transfected cells. Upon stable integration of pGTRTL regulation of luciferase expression was in excess of 60 fold for the highest expressing clones.

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Regulated expression of sCR1 from permanent transfectants with the construct GTRTC displayed maximal regulation in excess of 70 fold. The improved regulation observed with permanent transfectants probably occurs via favourable interactions with DNA sequences adjacent to the site of integration which reduce basal expression and enable good induction of gene expression.

25

The 'on'/'off' kinetics of gene expression was examined in stable DTF transfectants of GTRTL and GTRTC. In both cases gene expression reached or approached a maximum after 48 hours induction with Dox, and where Dox was removed from maximally induced cells expression approached or returned to baseline by 48 hours.

The kinetics of induction for these clones is slightly longer than reported for the permanently integrated two plasmids 'on' system where maximal expression is achieved in less than 24 hours in HeLa cells (Gossen *et al. Science*; **268**: 1766-1769 (1995)). The slower induction observed with our plasmids is probably explained by 5 the autoregulated expression of rtTA resulting in a delay in optimal levels of expression.

Autoregulated expression of rtTA in the 'on' system results in low basal levels of rtTA expression in the absence of Dox, and induction with Dox leads to increased levels of 10 rtTA through positive feedback. In this scenario high levels of rtTA are only produced when gene expression is induced with Dox. Therefore, gene therapy applications using this type of construct where short-term gene induction is required will ensure that cells are only exposed to high levels of rtTA for a short period of time. In addition the rapid kinetics of the switch 'off' observed in this study indicate that cells 15 will not be exposed to high levels of rtTA for prolonged periods of time following removal of Dox.

When DTF were co-transfected with the autoregulatory plasmid pGTRTC, and the 20 inducible plasmid pGTE, Dox inducible EGFP expression was detected in clones bearing the GTRTC plasmid. The regulation of sCR1 expression was of a similar magnitude to that observed with clones transfected with GTRTC alone. The expression of EGFP and sCR1 was shown to correlate over a time course up to 72 hours for one clone examined. These findings indicate that selection of clones on the 25 basis of their EGFP expression profile is a viable short cut in the selection of clones which display optimal regulatory expression of a gene of interest (GOI). Monitoring EGFP expression would be advantageous where rapid methods are not available to efficiently monitor expression of the GOI.

Example 3-TetP studies

The degree of gene regulation has been shown to vary considerably between different cell lines with limited regulation observed in some cells (Howe *et al.*, *J. Biol. Chem.* ; 5 **270**: 14168-14174 (1995)). It has been shown that tetP itself displays high basal promoter activity in DTF (mouse embryonic fibroblasts) (Gould *et al.* *Gene Therapy*; 7: 2061-2070 (2000)). High basal activity of the tetP has also been reported by other groups in several cell types including human cell lines following transient transfection (Freundlieb *et al.*, *J. Gene Med.*; **1**: 4-12 (1999)).

10

The DNA sequence of the tetP was examined for the presence of endogenous transcription factor binding sites which may contribute to the level of basal activity. The presence of the GATA binding motif WGATAR (where W is A or T and R is A or G) located within the tetR consensus sequence TCCCTATCAGTGATAGAGA 15 was identified. Six GATA transcription factors were identified, several of which participate in embryonic tissue differentiation (Maeda *et al.*, *J. Exp. Biol.* **20** (199):513-520 (1996)). In adult tissues GATA 1 to 3 are primarily associated with haematopoietic cells and GATA 4 to 6 are expressed in several organs including heart, intestine, ovaries, stomach and liver differentiation (Maeda *et al.*, *J. Exp. Biol.* **20** 20 (199):513-520 (1996)).

20

The interaction of GATA factors 4 to 6 with the tetP was assessed by co-transfection of an expression plasmid encoding a GATA factor and the tetP containing reporter plasmid pGTL. In addition tetP function in the presence of both a GATA factor and rtTA was assessed to determine additive of inhibitory effects between the transcription 25 factors.

MethodsDNA and Cells

30

The eukaryotic expression vectors mGATA-4pcDNA3, mGATA5pcDNA3 and mG6*PLINK which encode mouse GATA 4, GATA 5 and GATA 6 respectively were

provided by Prof. Roger Patient, Nottingham University. The retroviral vector ApHRO expressing mouse interferon β (Triantaphyllopoulos *et al.*, *Gene Therapy*; **5**: 253-263 (1998)) and the control vectors pcDNA3 (Invitrogen, Leek, The Netherlands) and pcEGFP from which EGFP is expressed from a CMV promoter were also used in
5 this study.

Cos 7 and DTF cells were cultured in DMEM supplemented with 10% FCS, glutamine (2 mM) and penicillin (100 U/ml) and streptomycin (100 g/ml).

10 Transfections. Transient transfections of Cos 7 cells plated at 2.5×10^5 on 12 well plates were performed by the calcium phosphate precipitation method described in Example 2. pGTL (5 μ g/well) was co-transfected with a GATA expression vector at a ratio of 1:1 (in each instance the total amount of DNA was adjusted with empty pcDNA3 plasmid to a molar equivalent of 15 μ g of the plasmid pGTL). A triplicate
15 transfection was also performed with GTL (5 μ g), pUHG 17.1 (encoding rtTA) and a GATA expression vector at a ratio of 1:1:1.

Results

20 Co-transfection of the tetP responsive reporter plasmid pGTL and each GATA expression vector demonstrated a degree of transcriptional activation by GATA 5 equivalent to a 5 fold increase in luciferase expression when transfected at a ratio of 1:1. GATA 4 and 6 did not induce luciferase expression. Luciferase expression was induced 62 fold by rtTA in the presence of Dox as shown in Figure 8.

25 Triplicate transfections with pGTL, pUHG 17.1 and a GATA expression vector in the presence of Dox demonstrated inhibition of luciferase expression by GATA factors. Compared to luciferase expression from pGTL induced by rtTA in the presence of Dox (6.79 RLU/ μ g protein (which represents 100%)), the addition of GATA 4, GATA 5 or GATA 6 reduced luciferase expression to 0.26, 0.47 and 4.38 RLU/ μ g protein as

shown in Figure 9. For GATA 4, GATA 5 and GATA 6 this represented an inhibition of luciferase expression equivalent to 98%, 95% and 36% respectively.

Conclusions

These observations indicate potentially important interactions between endogenous GATA factors and tetP. Transcriptional activation demonstrated by GATA 5 could contribute to increased basal activity of the tetP, whilst the significant inhibition of the Tet-regulatory system observed with GATA 4 and GATA 5 could clearly compromise the magnitude of gene regulation achieved by the system. Further studies will establish direct binding of GATA factors to the tetO and determine the influence of the other GATA factors (1 – 3) and interactions with the GATA transcriptional cofactors friend of GATA (FOG) 1 and 2 (Fox *et al.*, *EMBO J.*; **18**: 2812-2822 (1999)) on Tet regulated gene expression. Intriguingly many cells in which the Tet-regulatory system functions poorly are from embryonic tissue (HEK 293, NIH 3T3, DTF) and other GATA rich tissues such as ovaries (CHO cells) and haematopoetic cells (Jurkats). Indeed the embryonic fibroblast cell line NIH 3T3 has been shown to express mRNA for GATA 6 (Morrisey *et al.*, *Dev. Biol.*; **177**: 309-322 (1996)).

Construction of an enhanced tetP devoid of GATA binding sites but retaining a suitable recognition sequence for tetR is likely to result in improved Tet regulated gene expression in a wider range of cells and tissues. Previous studies on tetR binding to the tetO have revealed that base pair exchange of the GATA sequence in the tetO to TATA or GATC does not adversely affect gene repression in bacteria (Sizemore *et al.*, *Nuc. Acid Res.*; **18**: 2875-2880 (1990)), yet these or other substitutions could prevent GATA factors binding in mammalian cells.

Moreover, modification of the tetP to delete the interferon response elements (IREs) located between the tetO repeats is likely to result in improved Tet regulated gene expression. The IREs located between the tetO repeats are responsive to interferon stimulation and have been demonstrated in the 'off' system (Rang & Will *Nuc. Acid*

Res.; 28: 1120-1125 (2000)) and 'on' system (see Figure 10) and to increase basal activity of the tetP. In the context of the autoregulatory plasmid pGTRTL, IFN β expressed from ApHRO increases basal luciferase expression by four fold. This induction with IFN β remains functional in the dox induced state with an observed 5 magnitude of induction of 88 fold compared to 39 fold in the absence of IFN β .

Example 4 - Polyadenylation signal studies

The autoregulatory vectors described in the previous Examples are constructed around 10 the bidirectional polyadenylation signal (PolyA) from SV40. The polyadenylation sequence from SV40 is most efficient in the late orientation, with approximately five times higher levels of mRNA produced compared to its identical usage in the reverse or early orientation (Carswell & Alwine, *Mol. Cell. Biol.* 9: 4248- 4258 (1989)). In the autoregulatory vectors described in the previous Examples, the late Poly A 15 functions for the rtTA gene whilst the gene of interest (GOI) is terminated by the early PolyA. Preferably, the level of rtTA expression should be optimal to maximise regulation of the plasmid in the induced state.

The effect on the degree of gene regulation by reversing the SV40 PolyA for the rtTA 20 gene to the early sequence is described.

Methods

Cloning The terminal portion of the Luc+ and downstream late PolyA was isolated from pGL3 Basic (Promega Corp., Madison, USA) by restriction with PpuM I – 25 BamH I as shown in Figure 11. Similarly, restriction of pGTRTL with PpuM I and partial restriction with BamH I enabled the removal of a portion of the Luc gene, the 3'UTR and the early PolyA as shown Figure 11. Ligation of the insert from pGL3 and pGTRTL vector formed the construct, pGTRTL*, shown in Figure 11.

Transfection Human neuroblastoma NB 100 cells were plated on 6 well plates at 2.5 x 10⁶ cells/well and were transfected with 5µg per well of pGTRTL or pGTRTL* using the calcium phosphate precipitation method set out in Example 2. Cells were cultured with or without Dox (1 µg/ml) for 72 hours and then lysed and assayed for luciferase 5 activity.

Results

10 Luciferase expression from the non-induced pGTRTL* was found to be increased 7 fold above the basal expression from pGTRTL as shown in Figure 12. Induction with Dox for 72 hours lead to a 6 fold induction of luciferase expression from pGTRTL*. This 6 fold induction level is significantly higher than the Dox induced level from pGTRTL.

Discussion

15 The elevation in basal luciferase expression from pGTRTL* compared to pGTRTL was shown to be in part attributed to use of the late polyA for this gene as opposed to the early polyA in the latter plasmid. However, other factors may contribute to the increase in this basal luciferase expression. Firstly, removal of the 3'UTR derived 20 from the SV40 small t antigen located downstream from luciferase in pGTRTL may have the effect of increasing gene expression due to the removal of the potential inhibitory effect of 3'UTR (Evans & Scarpulla, *Gene*; **84**: 135-142 (1989)) and/or prevention of aberrant splicing caused by the 3'UTR (Huang & Gorman, *Mol. Cell Biol.*; **10**:1805-1810 (1990)).

25 Secondly, the luciferase gene in pGTRTL* is a fusion of the 5' section of the luciferase gene derived from pGL2 Basic (Promega Corp., Madison, USA) and the 3' section of the luciferase+ gene derived from pGL3 Basic. The 3' terminus of the luciferase+ gene has been modified to target the protein to the cytoplasm of the cell as 30 opposed to peroxisome targeting of the original luciferase protein (Sherf & Wood, *Promeg Notes*; **49**: 14 (1994)). Cytoplasmic targeting of luciferase results in a 4 to 5

fold increase in luciferase activity in transfected NIH 3T3 cells (Sherf & Wood, *Promeg Notes*; **49**: 14 (1994)) which may contribute, therefore, to the observed increase in expression.

5 The lower rtTA expression in pGTRTL* resulted in an overall lower degree of Dox induction compared to pGTRTL (6 versus 16) which indicates that the orientation of the SV40 PolyA in pGTRTL is optimal for gene regulation in NB 100 and other cells (preliminary data for Cos 7 and NIH 3T3 cells). Regulation may, however, be enhanced through the use of a more efficient PolyA signal than the late SV40 for the 10 rtTA gene, such as the bovine growth hormone PolyA (Yew *et al.*, *Human Gene Therapy*; **8**: 575-584 (1997)), although this would preferably be used in combination with a second PolyA in order to be bidirectional.

CLAIMS

1. An autoregulatory expression vector comprising a first nucleic acid construct and a second nucleic acid construct, the first and second nucleic acid construct comprising a promoter sequence and a tet-operator sequence (tetP); wherein, the first nucleic acid construct comprises a first nucleic acid sequence encoding a protein of interest and the second nucleic acid construct comprises a second nucleic acid sequence encoding reverse tetracycline transactivator (rtTA); and wherein each of the first and second nucleic acid sequences are provided with a termination sequence.
2. An autoregulatory expression vector as claimed in claim 1 which is a nucleic acid vector.
3. An autoregulatory expression vector as claimed in claim 2 wherein the nucleic acid vector is a bacterial plasmid.
4. An autoregulatory expression vector as claimed in claim 3 wherein the bacterial plasmid is pGTRTL.
5. An autoregulatory expression vector as claimed in claim 2 wherein the nucleic acid vector is a retrovirus.
6. An autoregulatory expression vector as claimed in any one of the preceding claims wherein the promoter comprises the minimum sequence for promoter activity.
7. An autoregulatory expression vector as claimed in claim 6 wherein the promoter is mCMV promoter.

8. An autoregulatory expression vector as claimed in any one of the preceding claims wherein the promoter is a mammalian promoter.

9. An autoregulatory expression vector as claimed in any one of the preceding claims 5 wherein the first nucleic acid sequence encodes a therapeutic protein.

10. An autoregulatory expression vector as claimed in claim 9 wherein the therapeutic protein is one or more of cytokine, growth factor, differentiation factor, receptor or antibody.

10 11. An autoregulatory expression vector as claimed in claim 10 wherein the therapeutic protein is soluble complement receptor 1.

15 12. An autoregulatory expression vector as claimed in any one of the preceding claims wherein the termination sequence is a bidirectional termination signal .

13. An autoregulatory expression vector as claimed in claim 12 wherein the termination signal is located between the first and second nucleic acid sequence.

20 14. An autoregulatory expression vector as claimed in any one of the preceding claims wherein the first tetP is in sense orientation and the second tetP is in antisense orientation.

25 15. An expression system comprising an autoregulatory expression vector as claimed in any one of claims 1 to 14.

16. An expression system as claimed in claim 15 which comprises one or more cells.

30 17. An expression system as claimed in claim 16 comprising one or more mammalian cells.

18. A method of treatment comprising administering to a recipient an autoregulatory expression vector as claimed in any one of claims 1 to 14 or an expression system as claimed in any one of claims 15 to 17.

5 19. A method as claimed in claim 18 wherein the treatment is the treatment of a chronic condition having relapsing symptoms.

10 20. A method as claimed in claim 18 or claim 19 wherein the treatment is gene therapy.

15 21. Products containing an autoregulatory expression vector as claimed in any one of claims 1 to 14 and a further expression vector comprising a second DNA molecule as a combined preparation for simultaneous, separate or sequential use in gene therapy.

22. Products according to claim 21 wherein the second DNA molecule is a nucleic acid vector.

20 23. Products according to claim 21 or claim 22 wherein the second DNA molecule comprises a nucleic acid sequence encoding a protein of interest.

24. An autoregulatory expression vector as claimed in any one of claims 1 to 14 or an expression system as claimed in any one of claims 15 to 17 for use in medicine.

25 25. Use of an autoregulatory expression vector as claimed in any one of claims 1 to 14 or an expression system as claimed in any one of claims 15 to 17 in the manufacture of a medicament for the treatment of a genetic disorder.

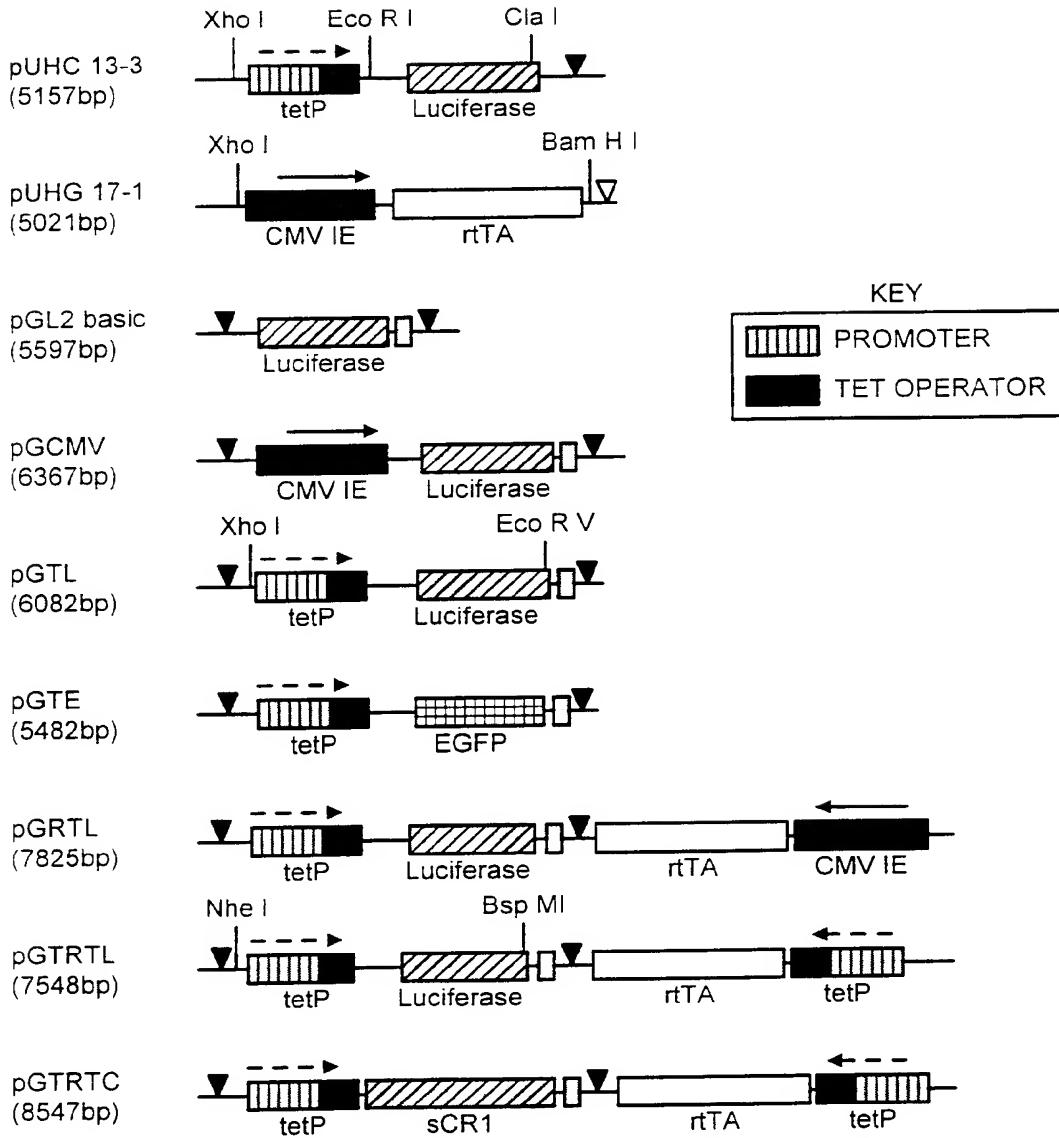
26. A pharmaceutical formulation comprising a pharmaceutically acceptable carrier and an autoregulatory expression vector according to any one of claims 1 to 14, an expression system as claimed in any one of claims 15 to 17 or products as claimed in any one of claims 21 to 23.

5

27. A kit of parts comprising an autoregulatory expression vector as claimed in any one of claims 1 to 14, an expression system as claimed in any one of claims 15 to 17 or products as claimed in any one of claims 21 to 23 and an administration vehicle.

10

A-Plasmid Vectors



B - Retroviral Vectors

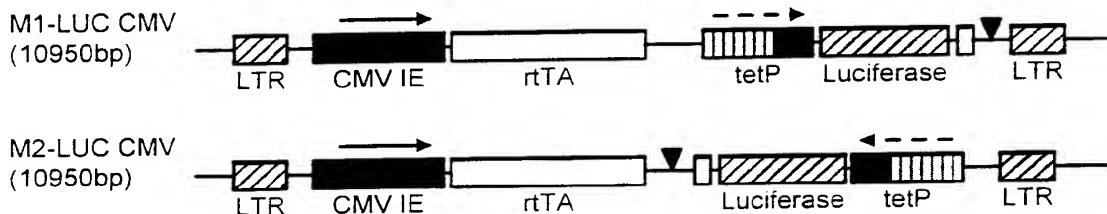


FIG. 1

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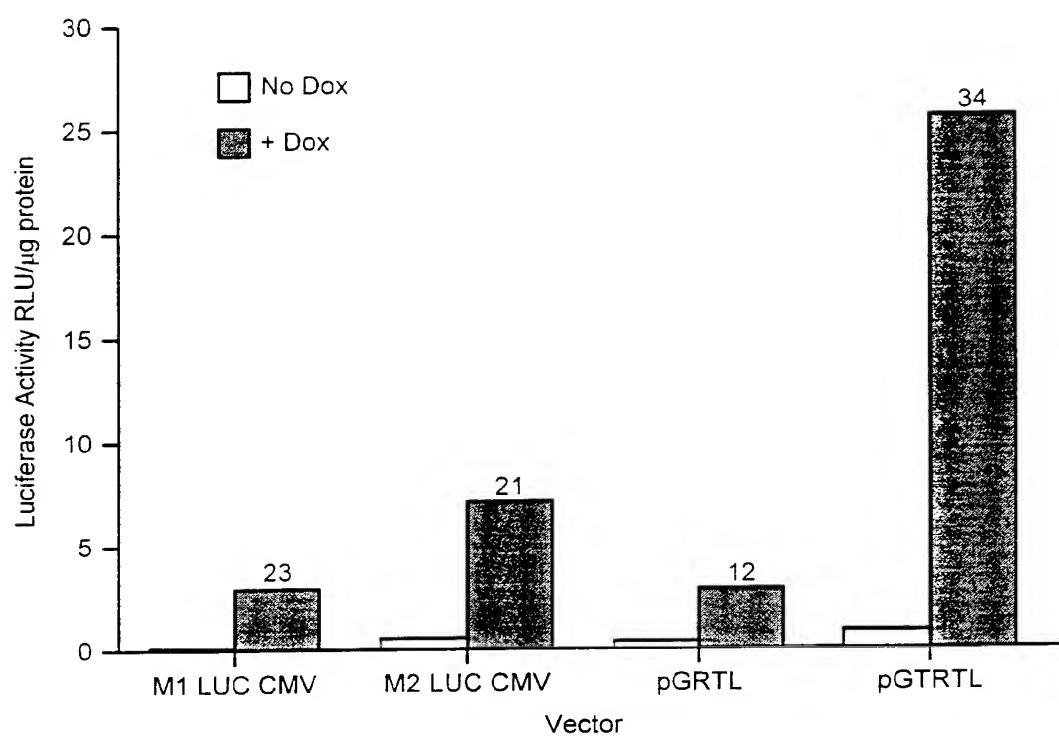
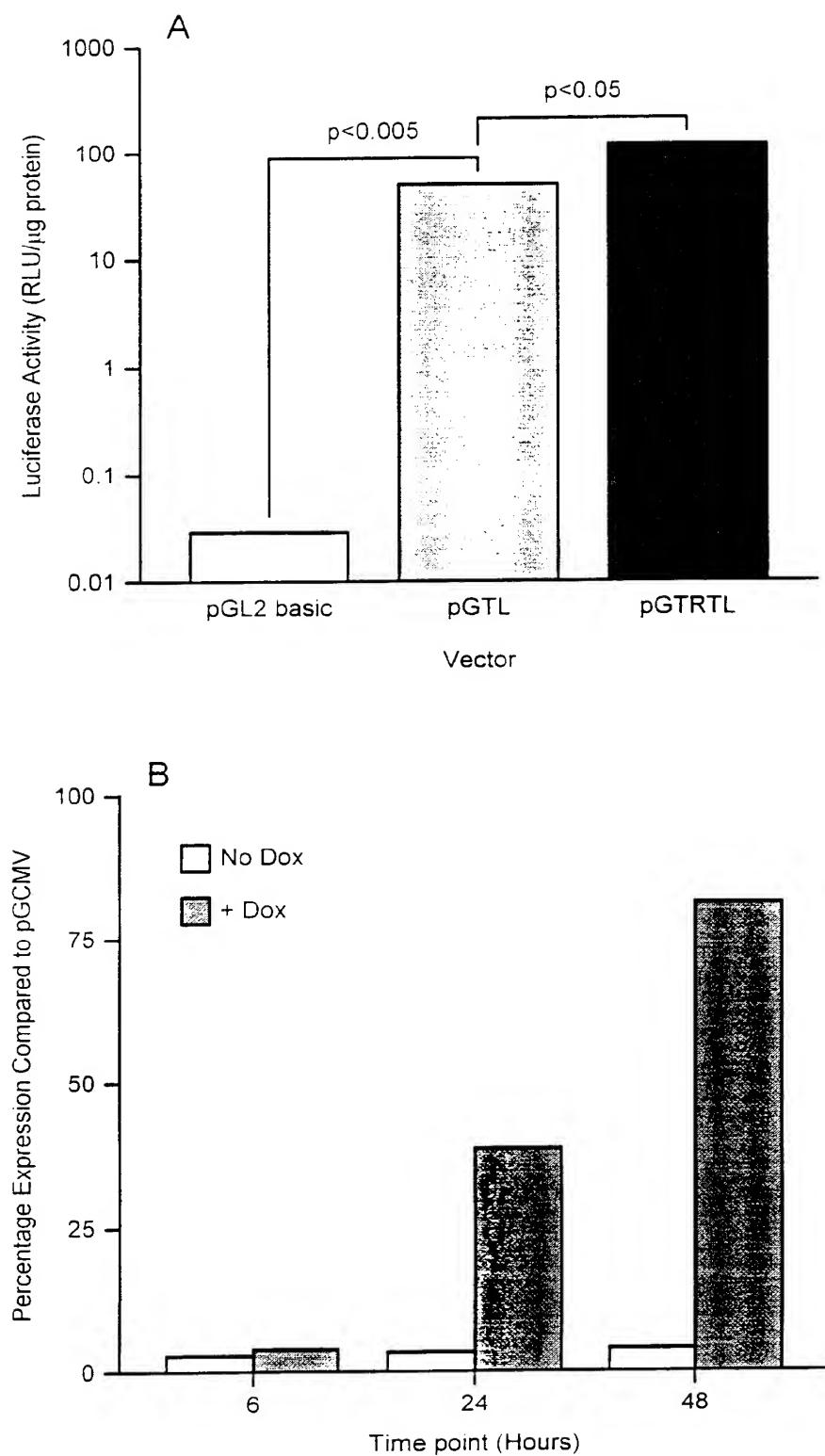


FIG. 2

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**FIG. 3**

4 / 12

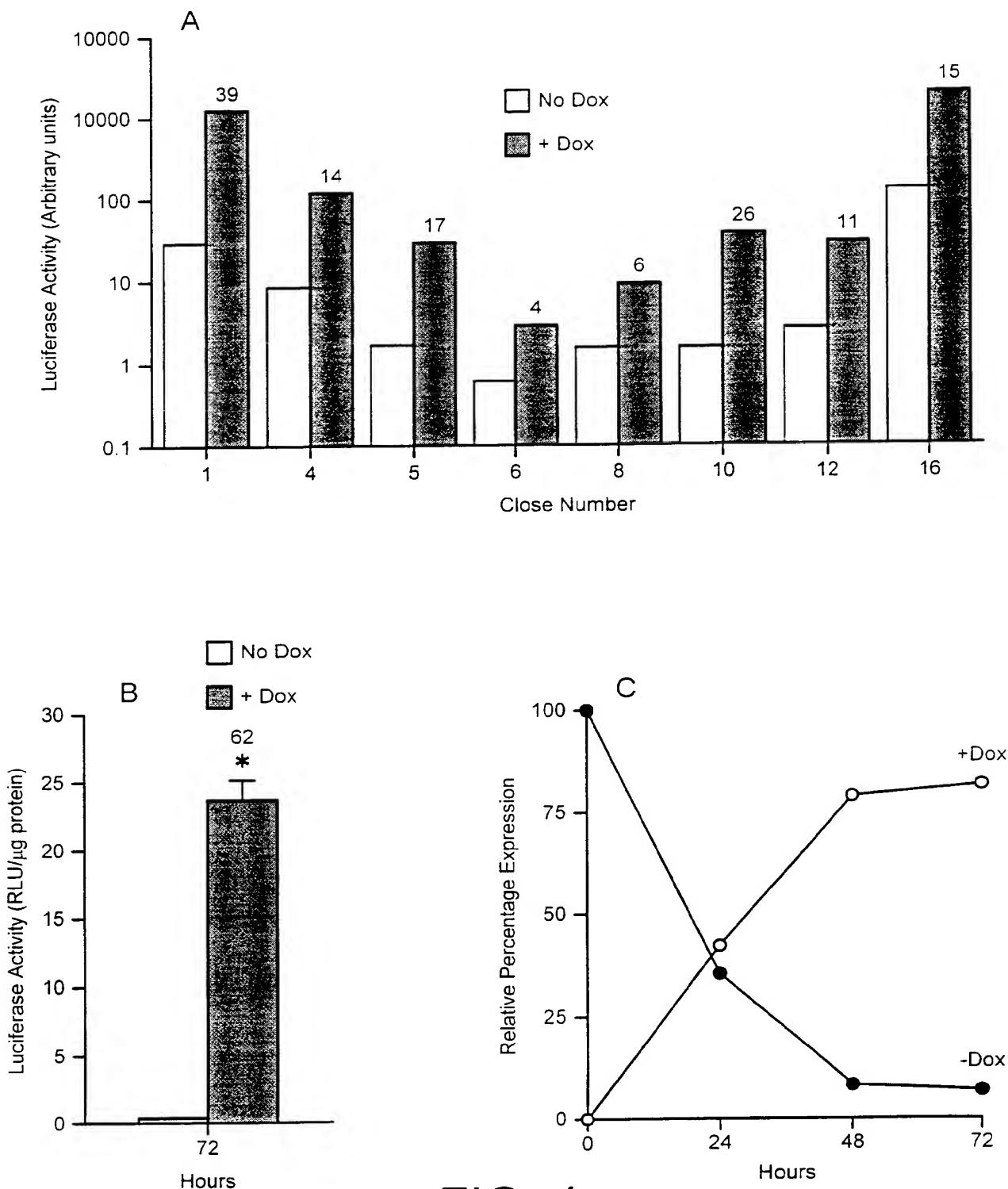


FIG. 4

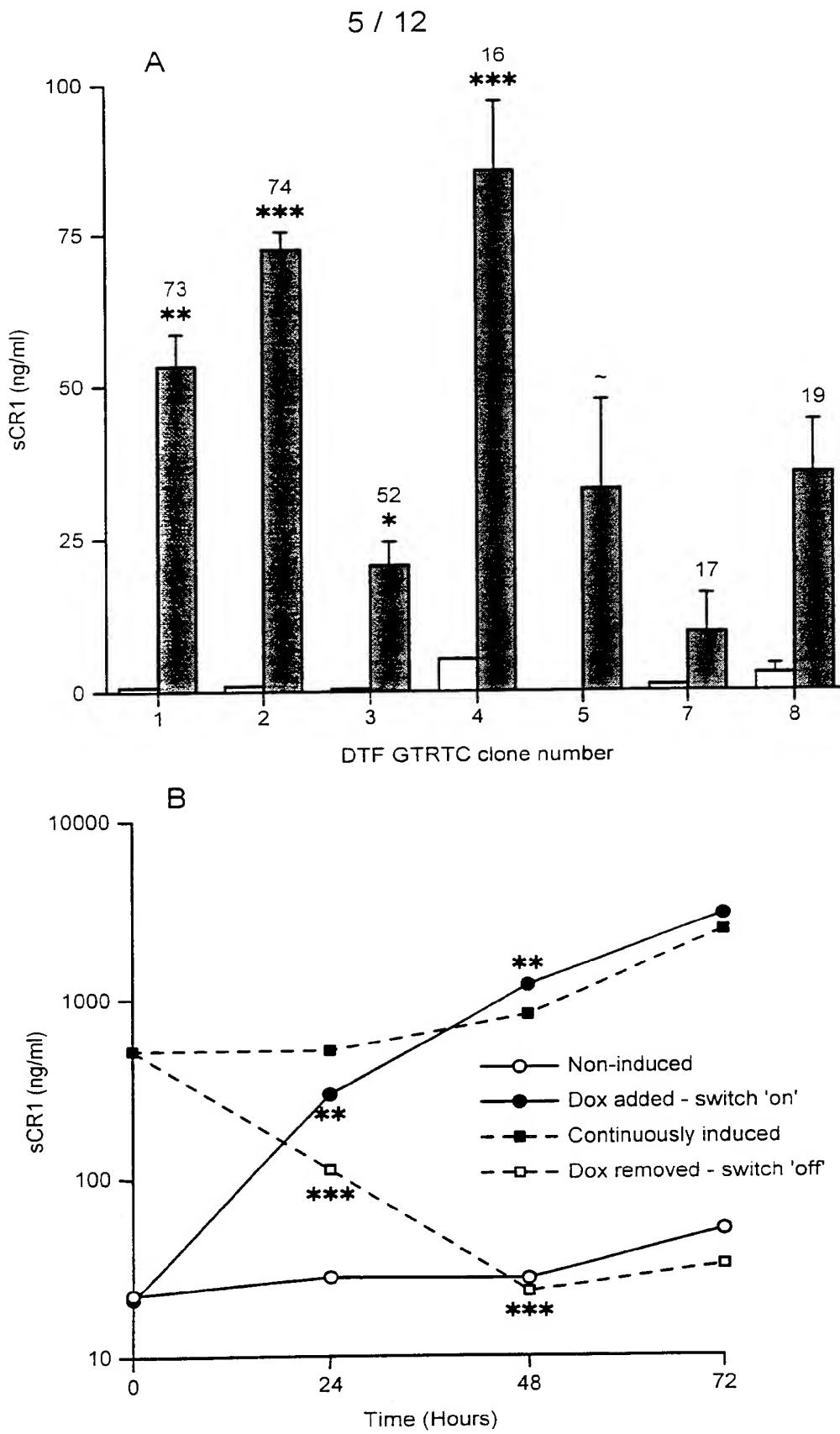


FIG. 5
SUBSTITUTE SHEET (RULE 26)

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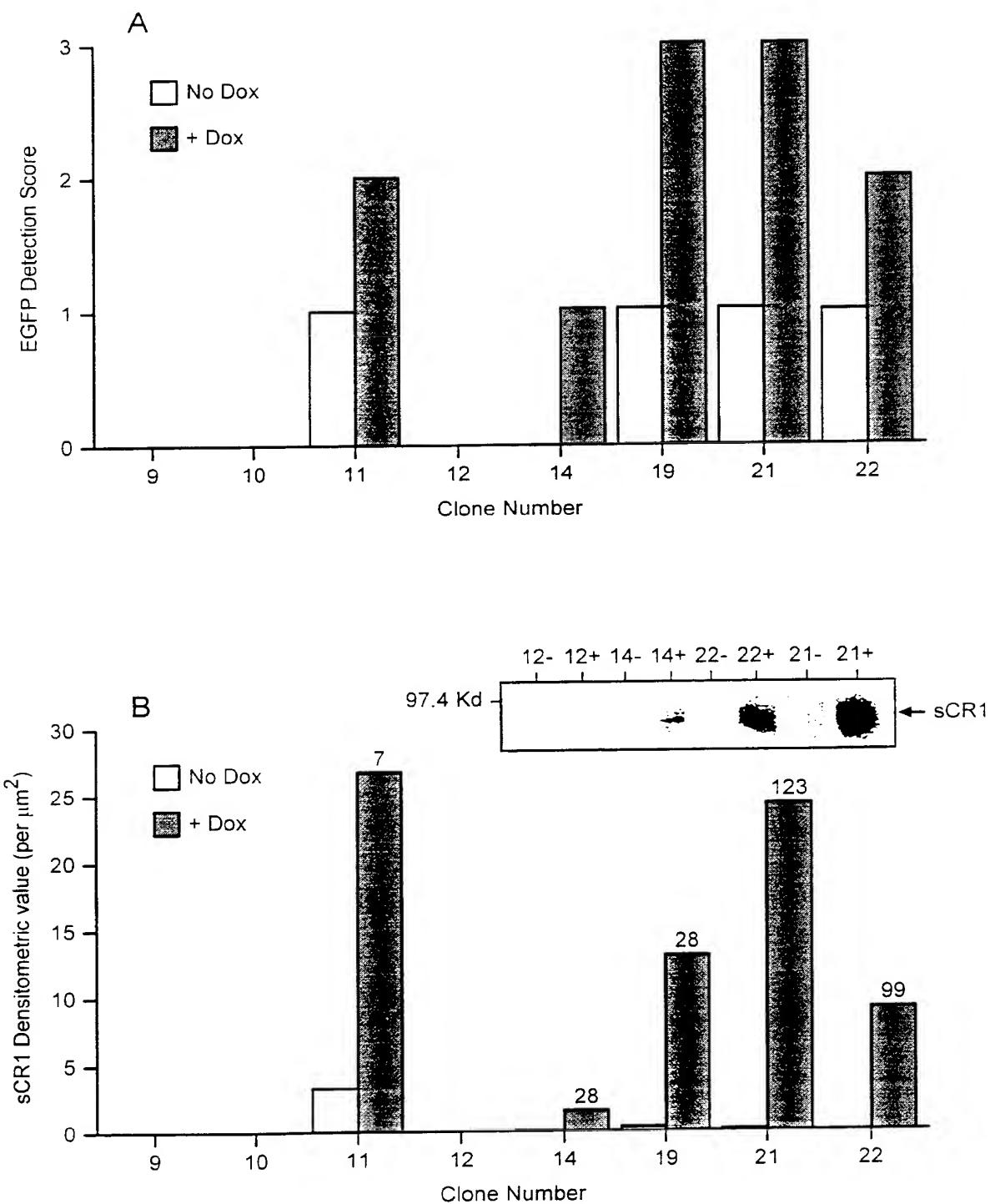


FIG. 6

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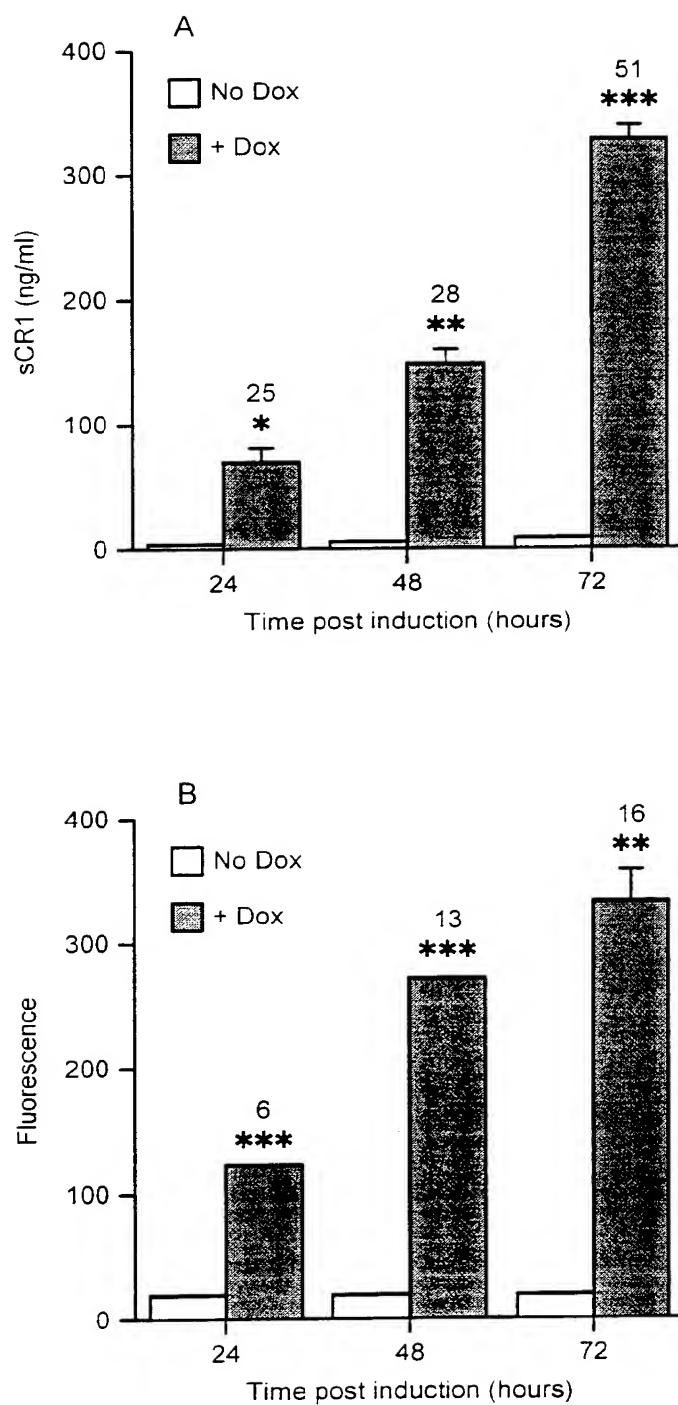


FIG. 7

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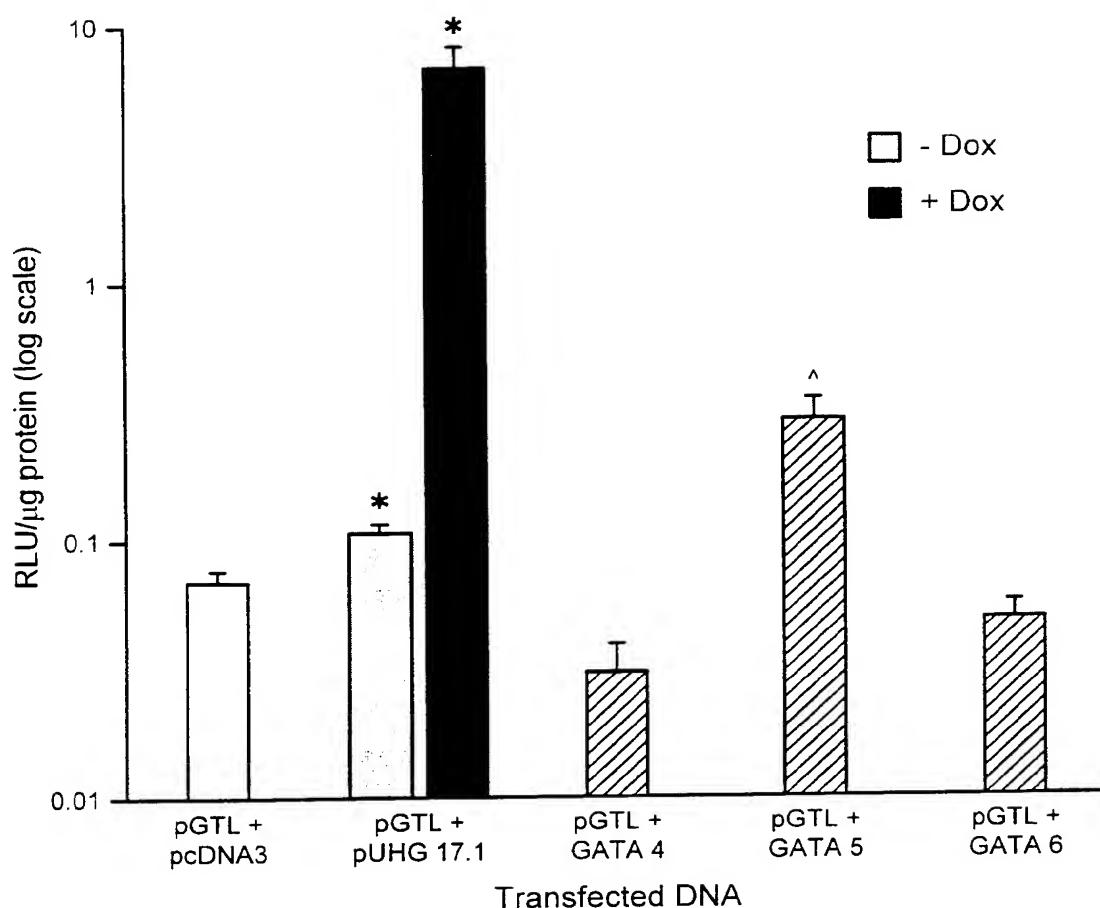


FIG. 8

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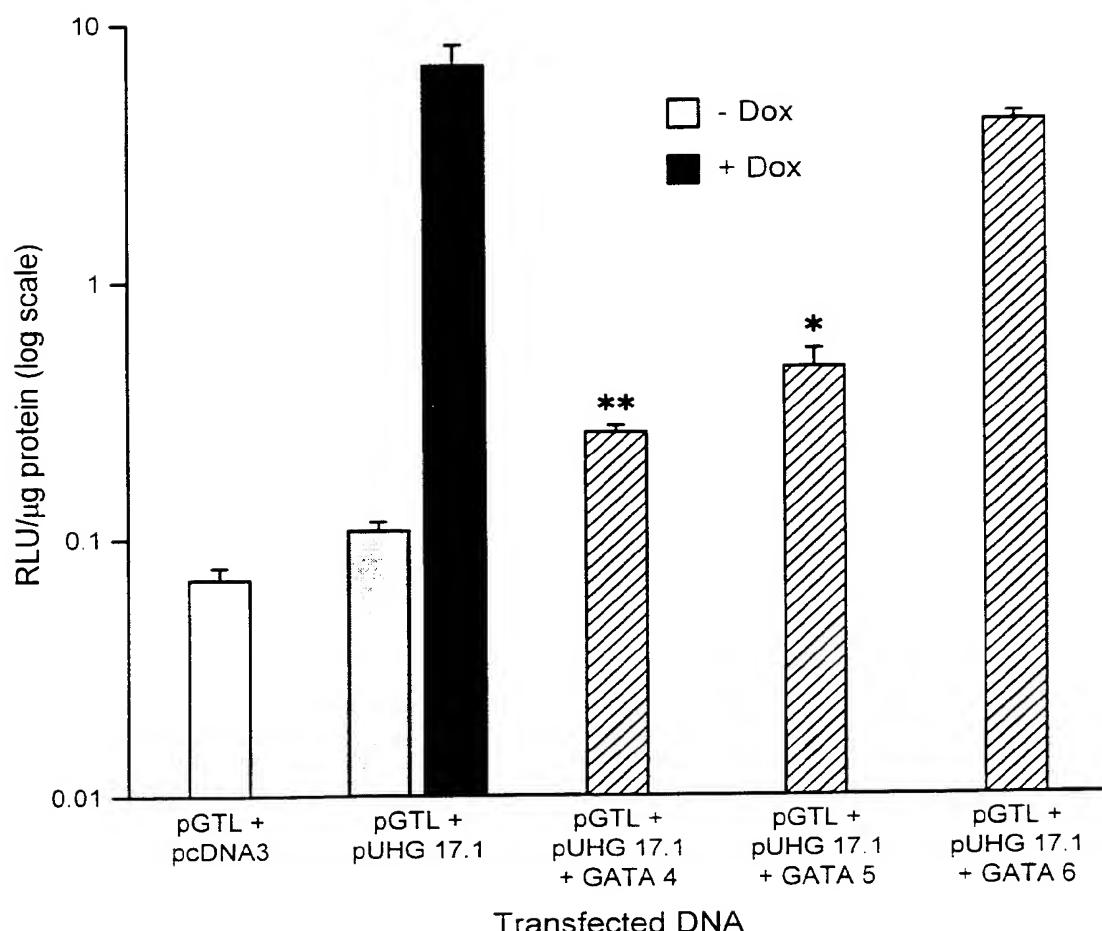


FIG. 9

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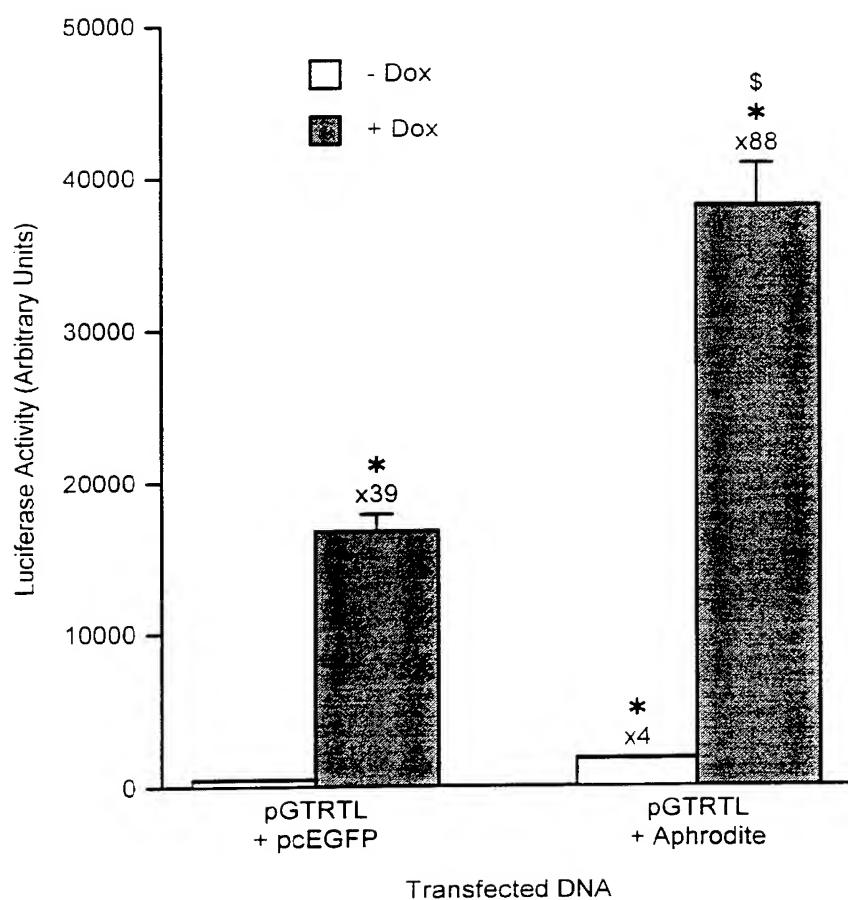


FIG. 10

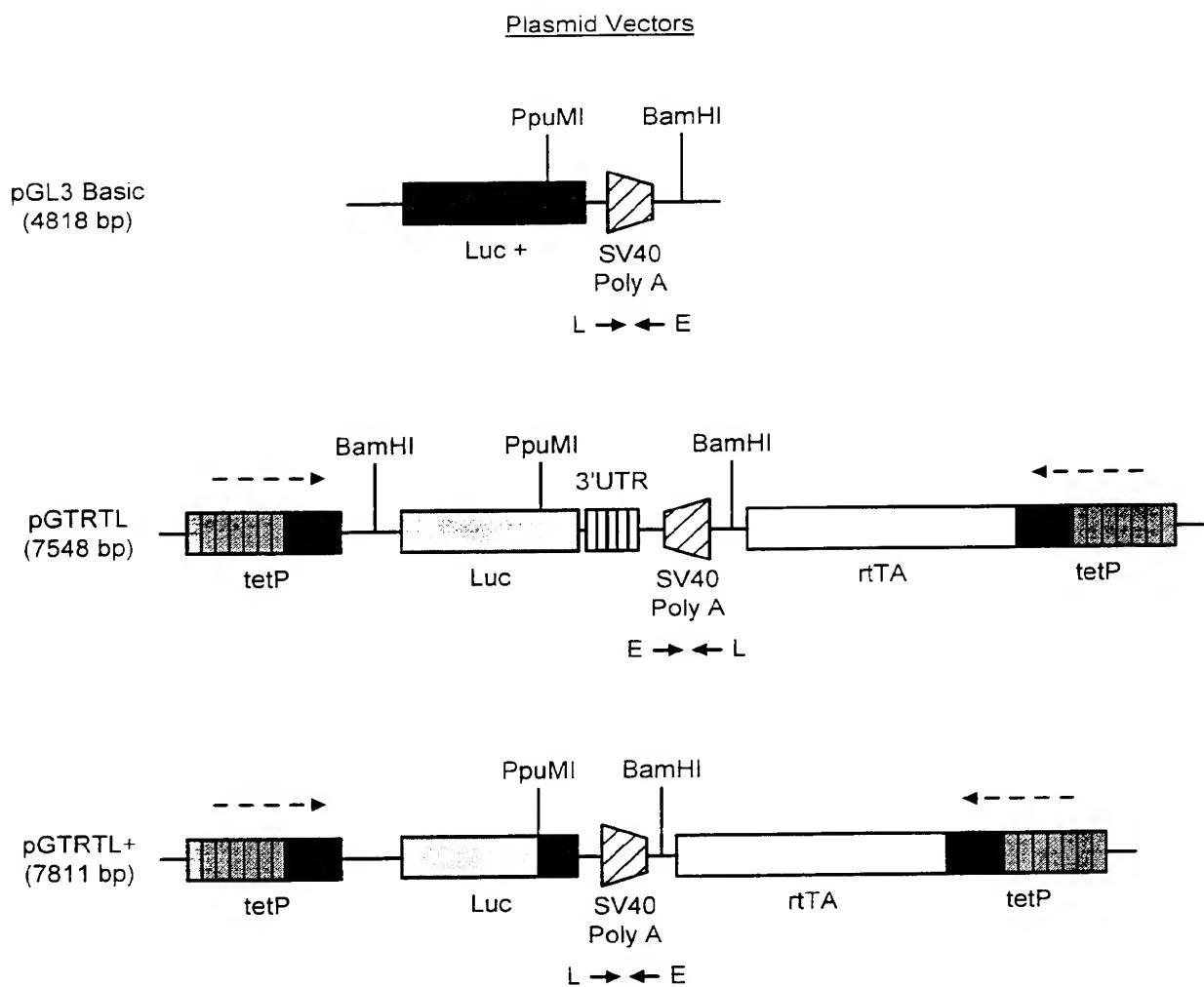


FIG. 11

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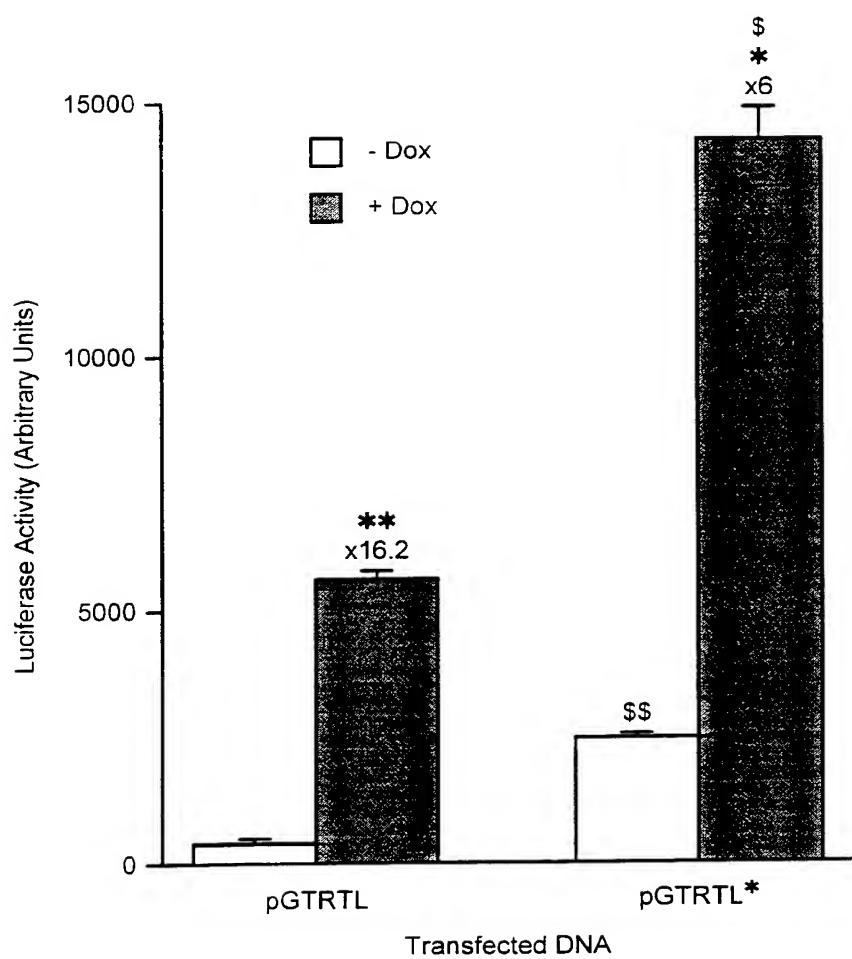


FIG. 12